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AWARD NUMBER DAMD17-97-1-7290

TITLE: ErB-2/HER2 Oncogene in Breast Cancer: Does Bivalency of Growth Factors Drive Tumorigenicity Through Receptor Heterodimerization

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REPORT DATE: October 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1998	Annual (1 Oct 97 - 30 Sep 98)

4. TITLE AND SUBTITLE
ErB-2/HER2 Oncogene in Breast Cancer: Does Bivalency of Growth Factors Drive Tumorigenicity Through Receptor Heterodimerization

5. FUNDING NUMBERS
DAMD17-97-1-7290

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Weizmann Institute of Science
Rehovot, Israel 76100

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The HER2/ErbB-2 oncogenic protein contributes to virulence of human cancer of epithelia cells, but the exact mechanism of its action is incompletely understood. We propose that ErbB-2 acts as a shared low affinity receptor for a large group of stroma-derived growth factors. In principle, we assume that the growth factors are bivalent and they recruit ErbB-2 through their low affinity sites. Evidence supporting this model has been obtained by analyzing several previously uncharacterized growth factors. In addition, mechanisms enabling enhancement of growth factor signaling by means of endocytosis and receptor degradation were uncovered. Molecular analyses of the ErbB-2 molecule by using specific antibodies and deletion mutants support direct binding of growth factors to ErbB-2 (albeit with low affinity) and provide initial structural data. Attempts to develop antagonists of the ErbB-2 binding sites are described

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	48
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Oct. 25, 1998

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Introduction

Background- One of the most significant prognostic factors of breast cancers is amplification and overexpression of the erbB-2/HER2 (also called neu) proto-oncogene. The encoded protein is a receptor-like tyrosine kinase whose exact mechanism of action is still unknown. Because ErbB-2 belongs to a family of growth factor receptors, that includes ErbB-1 (EGF-receptor) and two receptors for NDF/neuregulin (ErbB-3 and ErbB-4), it is thought that a still unknown ligand directly binds to it. However, despite extensive search, no such a ligand has been fully characterized. On the other hand, we have recently shown that ErbB-2 acts as a common auxiliary subunit of NDF- and EGF-receptors, that prolongs and augments growth factor responsiveness of breast cancer cells overexpressing ErbB-2. The augmenting action of ErbB-2 involves heterodimerization with other ErbB counterparts

Working hypothesis- We propose that all ligands of the NDF and EGF families are bivalent. Their high-affinity and narrow-specificity sites bind a primary receptor (ErbB-1, ErbB-3 or Erbb-4), whereas the distinct low-affinity and broad-specificity sites preferentially bind to ErbB-2. According to this model, ErbB-2 promotes tumorigenesis of tumor cells by acting as a low-affinity and broad-specificity receptor for all known ErbB ligands.

Experimental strategy- In an attempt to test the prediction that all ErbB ligands bind to ErbB-2 with low affinity and recruit it into heterodimeric receptor complexes we examined several previously untested ligands, including the two isoforms of the recently reported neuregulin-2 (NRG-2) ligands, epiregulin, and three Pox virud-encoded ligands, VGF, SFGF and MGF. An important implication of the bivalency model is the possibility that ErbB-2-containing heterodimers, due to their relatively high pH sensitivity, are driven into an endocytic pathway distinct from that of homodimers. This scenario was examined with three ErbB ligands: EGF, TGFα and NRG-1 (NDF-β). Intial experiments aimed at confirming the bivalence model with NDF have been carried out by using mutant forms of the ligand. Lastly, we addressed the prediction that ErbB-2 carries a ligand binding site by employing biophysical measurements, immunological

approaches that utilize antibodies to the putative site, and analysis of phage display libraries with the aim of identifying peptide antagonists.

Body of the Report

1. Do all ErbB ligands recruit ErbB-2 ? We have previously shown that ErbB-2 may function as a low affinity receptor of EGF and NDF. An essential component of our working hypothesis assumes that all ErbB ligands bind with low affinity to ErbB-2, and thereby they can recruit this oncogenic receptor into heterodimers. This prediction was tested in relation to several ligands that have not been examined before. These are NRG-2s, epiregulin, and three viral growth factors. The results are briefly summarized below. Full description is provided in two publications that are attached as Appendices.

(i) Nuuregulin-2 isoforms- The recently isolated second family of neuregulins, NRG-2, shares its primary receptors, ErbB-3 and ErbB-4, and induction of mammary cell differentiation, with NRG-1 isoforms, suggesting functional redundancy of the two growth factor families. To address this possibility, we analyzed receptor specificity of NRGs by using an engineered cellular system. Isoform-specific but partly overlapping patterns of specificities, that collectively activate all eight ligand-stimulatable ErbB dimers, was revealed. Specifically, NRG2-β, like NRG1-α, emerges as a narrow specificity ligand, whereas NRG2-α is a pan-ErbB ligand that binds with different affinities to all receptor combinations, including those containing ErbB-1, but excluding homodimers of ErbB-2. The latter protein, however, displayed cooperativity with the direct NRG receptors. This latter observation is consistent with a ligand bivalence model.

(ii) Epiregulin- W addressed the action of epiregulin, a recently isolated ligand of ErbB-1 that differentially affects epithelial cells (growth arrest or mitogenesis). By employing a set of factor-dependent cell lines engineered to express individual ErbBs or their combinations, we found that epiregulin is the broadest specificity EGF-like ligand so far characterized: not only does it stimulate homodimers of both ErbB-1 and ErbB-4, it also activates all possible heterodimeric ErbB complexes. Consistent with its relaxed selectivity, epiregulin binds the various receptor combinations with an affinity that is approximately 100-fold lower than the affinity of ligands with more stringent selectivity, including EGF. Nevertheless, epiregulin action upon most receptor combinations

transmits a more potent mitogenic signal than does EGF. This remarkable discrepancy between binding affinity and bioactivity is permitted by a mechanism that prevents receptor down-regulation, and results in a weak, but prolonged, state of receptor activation. Importantly, ErbB-2 is recruited quite efficiently into epiregulin-driven heterodimers, in line with our working hypothesis.

(iii) Viral ErbB ligands- Virulence of Poxviruses, the causative agents of smallpox, depends on virus-encoded growth factors related to the mammalian epidermal growth factor (EGF). We report that the growth factors of Shope fibroma virus, Myxoma virus, and vaccinia virus (SFGF, MGF and VGF) display unique patterns of specificity to ErbB receptor tyrosine kinases: whereas SFGF is a broad-specificity ligand, VGF binds primarily to ErbB-1 homodimers, and the exclusive receptor for MGF is a heterodimer comprised of ErbB-2 and ErbB-3. In spite of 10-1,000 fold lower binding affinity to their respective receptors, the viral ligands are mitogenically equivalent or even more potent than their mammalian counterparts. This remarkable enhancement of cell growth is due to attenuation of receptor degradation and ubiquitination, that leads to sustained signal transduction. Our results imply that signal potentiation and precise targeting to specific receptor combinations contribute to cell transformation at sites of Poxvirus infection, and they underscore the importance of the often ignored low affinity ligand-receptor interactions. Most important to our hypothesis, the finding that the extremely pathogenic virus, Myxoma virus, engages ErbB-2/ErbB-3 heterodimers in its route of infection suggest that ErbB-2 may not have a direct ligand. Instead, it acts as a shared low-affinity receptor for both mammalian and viral growth factors.

2. Implications of the bivalence model to mitogenic potency and intracellular sorting of ErbBs

Signaling by receptor heterodimers, and especially ErbB-2-containing heterodimers, is superior over homodimers. We addressed the mechanism underlying this superiority and its relationship to ligand bivalency by using three growth factors: EGF, transforming growth factor α (TGFα), and their chimera, denoted E4T, that act on cells singly expressing ErbB-1 as a weak, a strong, and a very strong agonist, respectively. Co-expression of ErbB-2 specifically potentiated EGF signaling to the level achieved by TGFα, an effect that was partially mimicked by ErbB-3. Analysis of the mechanism

underlying this trans-potentiation implied that EGF-driven homodimers of ErbB-1 are destined to intracellular degradation, whereas the corresponding heterodimers with ErbB-2, or with ErbB-3, dissociate in the early endosome. As a consequence, in the presence of either co-receptor, ErbB-1 is recycled to the cell surface and its signaling is enhanced. This latter route is followed by TGFα-driven homodimers of ErbB-1, and also by E4T-bound receptors, whose signaling is further enhanced by repeated cycles of binding and dissociation from the receptors. We conclude that alternative endocytic routes of homo- and hetero-dimeric receptor complexes may contribute to tuning and diversification of signal transduction. In addition, the ability of ErbB-2 to shunt ligand-activated receptors to recycling may explain, in part, its oncogenic potential.

3. Towards molecular definition of the putative ligand binding site of ErbB-2

(i) Mutagenesis of ErbB-2- Several ErbB-2 mutants have been constructed in order to address the location of the putative low-affinity ligand binding site of ErbB-2. These mutants are briefly discussed below:

(i) ErbB-2ΔI and ErbB-2ΔIII- ErbB-2 shares a common cellular architecture with the ErbB family of receptor tyrosine kinases: a glycosylated extracellular domain, an hydrophobic transmembrane region, and a cytoplasmic tail that contains the tyrosine kinase and autophosphorylation sites. The extracellular domain (650 amino acids) of ErbB-2 is divided into four sub regions (I to IV), II and IV are cysteine-rich domains (CRD1 and CRD-2). The binding cleft resides in domain I or in domain III of the receptor, but presumably not in the flanking cysteine-rich domains (CRDs). Two separate mutants were therefore prepared, ΔI and ΔIII, by using single-strand DNA and oligonucleotide-directed mutagenesis. The erbB-2 cDNA was inserted into pcDNA3, a plasmid vector that enables mutagenesis, and the mutations were constructed.

In order to determine whether the mutated receptors are expressed on the cell surface, cell surface biotinylation assays were performed. Confluent monolayers were biotinylated at 4°C for 30 min, and cell extracts subjected to immunoprecipitation with antibodies against ErbB-2. Biotinylated proteins were detected after blotting using streptavidin-peroxidase. To detect expression of the receptors, membrane filters were stripped and re-blotted using antibodies against ErbB-2. It was found that only a small

portion of the total mutant ErbB-2 protein is detected at the surface. Co-expression of ErbB-3 together with the ErbB-2 mutants did not improve surface expression.

Our previous work generated several tumor-inhibitory antibodies that were classified into different subgroups. Subtype II mAbs to ErbB-2 decrease binding of EGF and NDF to their receptors on culture cells, and reduce heterodimer formation. These observations suggested that type II antibodies inhibit the direct interaction of ligands with the ErbB-2 protein. We took advantage of deletion mutants ErbB-2ΔIII and ErbB-2ΔI to narrow the region of the ErbB-2 extracellular domain which is involved in binding of subclass II mAb. CHO cell lines expressing the two mutants of ErbB-2 were incubated for 16 h in ^{35}S-methionine-containing medium, and ErbB-2 mutant and wild type proteins immunoprecipitated using different mAbs against ErbB-2, as well as a rabbit antibody against the C-terminus of ErbB-2. It has been noted that whereas two strong tumor-inhibitory antibodies, N12 and L431 (subclass I) are directed to domain III, subclass II monoclonal antibodies are apparently directed to domain I. Thus, subclass II mAbs are directed to domain I of the ErbB-2 extracellular domain, implying that the ErbB-2ΔI mutant may enable blocking of cis-acting factors, sparing the trans-acting effects of ErbB-2. However, deletion of domain I, by itself, activates ErbB-2 phosphorylation, probably by de-inactivation of dimer formation.

Because mutants ErbB-2 proteins were largely entrapped within the cell we assume that interruption of the secondary structure (cysteine bridging and specific sugar residues) prevented targeting to the membrane. Nevertheless, our studies with ErbB-2 mutants suggest that the N-terminal domain of ErbB-2 fulfils the criteria for a low affinity ligand binding site. Our next approach will be to construct chimeric ErbB-proteins that include this domain of ErbB-2. We plan to construct chimeras with both ErbB-1 and ErbB-3 as proposed in our original grant application.

(ii) An immunological approach- A series of 22 monoclonal antibodies (mAbs) to the human ErbB-2 has been generated by immunizing mice with an Fc-ErbB-2 fusion protein. mAbs were first classified into several groups according to their antigenic epitopes. In addition, the ability of the mAbs to inhibit tumor growth in athymic mice was determined by using a human gastric cancer cell line, N-87. We found that several antigenic epitopes can mediate tumor inhibition. The most immunogenic epitope apperas to correspond to the putative low-affinity and broad-specificity site because antibodies

directed to this site can reduce the binding affinity of several growth factors by decelerating their rate of dissociation from the cell surface. In addition, mAbs directed to the putative binding site (denoted class II mAbs) could inhibit formation of heterodimeric complexes containing ErbB-2. As a consequence, class II mAbs also reduced the mitogenic potency of EGF and NDF when these ligands were tested with interleukin-dependent cells. Currently we concentrate on monovalent fragments of certain class II mAbs with the hope of developing antagonists of the putative common binding site of ErbB-2.

(iii) A biophysical approach- To directly examine the interactions of various growth facors with the extracellular domain of ErbB-2 we have undertaken a biophysical approach. NDF-β1 was covalently immobilized to dextran fibers of a Biacore (Pharmacia) flow cell and the kinetics of interaction with a soluble ErbB-2 derivative was studied by measuring changes in surface plasmon resonance of an underlying gold film. The soluble ErbB-2 derivative, a fusion protein between the extracellular domain of ErbB-2 and the Fc portion of human IgG_1 (denoted IgB-2) was injected at various concentrations into the flow cell and kinetic constants calculated by using Biacore Incorporated software. As controls, we performed the same analysis with similar IgG fusion versions of the other three ErbB proteins (receptorbodies). As expected, soluble forms of the two direct NDF receptors (IgB-3 and IgB-4) displayed similar rapid on rates and slow off rates when analyzed on an immobilized NDF, yielding K_D values that are consistent with previously determined parameters for soluble or membrane-bound receptors. However, NDF interacted, albeit weakly, also with a soluble ErbB-2, as well as with a soluble ErbB-1. Whereas both receptors associated with the immobilized ligand at a similar rate, that was approximately 50-fold slower than that displayed by the direct receptors, their dissociation rates differed: ErbB-2 released NDF relatively slowly. The calculated affinity of ErbB-2 to NDF was 0.85 μM, unlike the 10^{-9}M values that were displayed by the direct receptors, ErbB-4 and ErbB-3. Our plan is to reproduce these measurements with mutants of ErbB-2, especially those that are deleted at domain I (see above description).

4. Attempts to generate antagonists of the putative ErbB-2 binding site

Because of the preliminary nature of our attempts to direct molecular reagents to the putative low-affinity/broad-specificity site of ErbB-2, these studies will be described only in brief. The following approaches are being taken and their results summarized below:

(i) Class II mAbs- Monovalent fragments of class II mAbs to the human ErbB-2 were generated and initially tested in vitro. We identified some Fab fragments that retained their high affinity binding to surface-expressed ErbB-2. As predicted, these fragments could accelerate dissociation of EGF and NDF from the surface of ErbB-expressing cell lines, implying that they may act as ErbB-2 antagonists. Their potential anti-tumor activity is currently being examined.

(ii) Mutants of NDF- We aim at generating mutant forms of growth factors whose ability to recruit ErbB-2 is defective. Thereby, such mutants may act as antagonists of ErbB ligands. To this end we are generating point mutations at both the N- and C-terminal tails of NDF (Leu3, Val4 and Val23, as well as Val49 and Met50), two sites that presumably carry the high and low affinity binding function, respectively. In addition, we undertake an alternative approach that involves generation of short analogs of NDF, namely: the N-terminus fused to the C-terminal 5 amino acids through a linker whose structure conforms to the predicted 3D structure. Results obtained along these two lines of research will be described inn our next report.

(iii) Screening of phage display libraries- To isolate peptide binders of ErbB-2 we undertake two approaches. First, a soluble form of ErbB-2 (a fusion between Fc and the ectodomain of the human ErbB-2) is being used to screen pahge display peptide libraries. Second, one of our type II mAbs (L26-IgG) is being used on the same libraries with the hope of isolating good binders that mimic the putative low-affinity/broad-specificity binding site of ErbB-2. So far we failed in isolating direct binders of ErbB-2. However, initial screening of a fd phage library that contains inserts of two 10 amino acid-long exons yielded several positive peptides. The corresponding phages were sequenced and the peptides synthesized . None of the peptides identified resembles the sequence of ErbB-2. Nevertheless, binding was antibody-specific as other types of mAbs did not bind to the peptides we synthesized. Competition analyses showed that the best peptide binder could inhibit only 50% of the binding of mAb L26, indicating low affinity of interaction. Therefore, we plan to construct constraint libraries in order to enhance peptide binding.

Conclusions

(i) Analyses of several ErbB ligands that have not been examined before provided further support to the hypothesis that ErbB-2 acts solely as a shared receptor subunit. Thus, none of the ligands we tested binds directly to ErbB-2, but all ligands recruit it into heterodimeric complexes. This extends also to viral ligands, implying that ErbB-2 evolved as a receptor whose specificity is relaxed, and therefore its affinity is low.

(ii) Two new mechnisms that allow ErbB ligands to enhance their mitogenic potential were identified. First, some ligands escape negative regulation by their inability to induce receptor endocytosis and degradation. Second, we found that ErbB-2-containing heterodimers, unlike ErbB-1 homodimers, are recruited to the recycling pathway, leading to prolonged signaling.

(iii) Biophysical and covalent crosslinking analyses also support a bivalence model of ErbB action. We found that ErbB-2 directly binds various ligands but with very low affinity.

(iv) Preliminary analyses suggest that the putative ligand binding site of ErbB-2 maps to the most N-terminal region of this receptor. However, because deletions impaired membrane targeting of mutant ErbB proteins and also activated the associated kinase activity we plan to further test this conclusion.

(v) We have so far failed in generating an anatagonist reagent that blocks ErbB-2 action. However, the finding that a certain class of mAbs can block the putative ErbB-2 ligand binding site may enable isolation of competitive peptide antagonists.

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MOLECULAR AND CELLULAR BIOLOGY, Oct. 1998, p. 6090–6101 Vol. 18, No. 10
0270-7306/98/$04.00+0

ErbB Tyrosine Kinases and the Two Neuregulin Families Constitute a Ligand-Receptor Network

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Received 21 August 1997/Returned for modification 21 October 1997/Accepted 7 July 1998

The recently isolated second family of neuregulins, NRG2, shares its primary receptors, ErbB-3 and ErbB-4, and induction of mammary cell differentiation with NRG1 isoforms, suggesting functional redundancy of the two growth factor families. To address this possibility, we analyzed receptor specificity of NRGs by using an engineered cellular system. The activity of isoform-specific but partly overlapping patterns of specificities that collectively activate all eight ligand-stimulatable ErbB dimers was revealed. Specifically, NRG2-β, like NRG1-α, emerges as a narrow-specificity ligand, whereas NRG2-α is a pan-ErbB ligand that binds with different affinities to all receptor combinations, including those containing ErbB-1, but excluding homodimers of ErbB-2. The latter protein, however, displayed cooperativity with the direct NRG receptors. Apparently, signaling by all NRGs is funneled through the mitogen-activated protein kinase (MAPK). However, the duration and potency of MAPK activation depend on the identity of the stimulatory ligand-receptor ternary complex. We conclude that the NRG-ErbB network represents a complex and nonredundant machinery developed for fine-tuning of signal transduction.

One of the relatively simple systems of signal transduction by a polypeptide growth factor is the mechanism controlling vulva formation in the nematode *Caenorhabditis elegans* (reviewed in reference 33). The most ancient epidermal growth factor (EGF)-like ligand, Lin-3, which is expressed by the anchor cell, binds to the Let-23 transmembrane tyrosine kinase on the surface of the closely apposed vulva precursor cell. The latter is then directed to a vulval fate through a biochemical cascade that sequentially activates a small GTP binding protein and a series of protein kinases, culminating in the mitogen-activated protein kinase (MAPK). A remarkably expanded version of this signaling module exists in mammals (reviewed in reference 6). Four receptors, whose structures are homologous to Let-23, and a few dozen ligands, all sharing the three-loop structure of EGF, form an interactive system with a large potential for signal diversification. In addition to the multiplicity of components, the modern version of the module is characterized by diversity: one ErbB protein, ErbB-3, is devoid of tyrosine kinase activity (25), and another, ErbB-2, binds no known EGF-like factor with high affinity (28, 61). Likewise, the various ligands carry, in addition to the EGF-like motif, a variety of structural domains thought to allow interaction with extracellular components. For example, the heparin binding EGF-like factor includes a heparan sulfate binding moiety (26), and the Neu differentiation factor (NDF, also called neuregulin 1 [NRG1], or heregulin) carries an immunoglobulin (Ig) domain (27, 37, 63).

A combination of in vitro experiments and gene targeting in mice implies that the mammalian ErbB module, like its invertebrate counterparts in worms and in flies (46), is involved with fate determination of several cell lineages. Thus, ErbB-1, and some of its ligands, control the development of specific types of epithelia (42), whereas NRG1 and its receptor, ErbB-4, play an essential role in formation of trabeculae in the embryonic heart (21, 41). Other functions of neuregulins include strengthening of the neuromuscular synapse (19); differentiation of myelin-producing cells, both Schwann cells (17) and oligodendrocytes (8); and lobulo-alveolar differentiation in the mammary gland (65). Each of these physiological roles depends on a specific combination of receptors, which likely represents the necessity for receptor heterodimerization, as opposed to homodimerization, for signaling. The importance of receptor heterodimerization, a process that does not exist in the invertebrate forms of the module, is exemplified by gene targeting of *erbB-2*: Despite the fact that this receptor has no direct ligand, the resulting phenotype is almost identical to those of *NRG1*- and *erbB-4*-targeted mice (35).

Through functional inactivation of ErbB-2 in cultured cells (4, 23, 24, 30) and ectopic expression of single or specific pairs of ErbB proteins in defined cellular contexts (11, 15, 49, 52, 62, 67), it became clear that the mammalian ErbB module functions as a signaling network. In general, homodimers of ErbBs are either devoid of biological activity (i.e., ErbB-3 homodimers) or are weakly active (e.g., ErbB-1 homodimers), and heterodimeric combinations are strongly active. Most potent are ErbB-2-containing combinations, whose signaling is prolonged because of an ErbB-2-mediated deceleration of ligand dissociation (30). Importantly, each ligand appears to be characterized by a distinct ability to stabilize specific homo- and heterodimeric receptors (48), thus enhancing the diversification potential of the network. According to a recently proposed model, ligand-specific dimerization is due to bivalence of EGF-like growth factors: their high-affinity site binds a pri-

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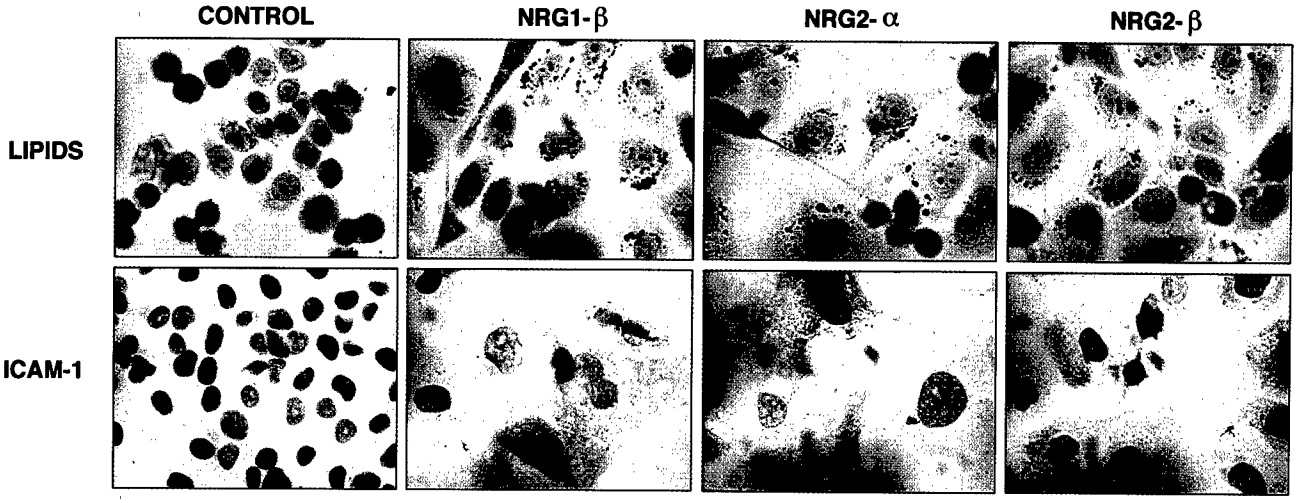


FIG. 1. Induction of cellular differentiation by neuregulin isoforms. AU-565 human mammary cancer cells, which express all four ErbB proteins, were plated in chamber slides and then incubated for 4 days in the absence (CONTROL) or presence of the indicated NRG isoforms (each at 50 ng/ml). Cells were stained with either Oil red O, to visualize neutral lipids, or with an antibody to ICAM-1. Antibody visualization was performed by using a biotinylated rabbit anti-mouse IgG, followed by an alkaline phosphatase-conjugated streptavidin and a red chromogen. Note the appearance of lipid droplets (yellow) and ICAM-1 (red stain) in NRG-treated cells. The magnification used was ×444 (lipid staining) or ×296 (ICAM-1 staining).

mary receptor (ErbB-1, -3, or -4), and a low-affinity site whose specificity is broad selects the interacting receptor with some preference for ErbB-2 (61).

On the basis of the lines of evidence described above, it seems safe to conclude that multiplicity of receptors and ligands increases the functional versatility of the mammalian ErbB signaling module. Therefore, the recent isolation of an additional family of EGF-like ligands of ErbB proteins, denoted NRG2 (7, 9, 12), is expected to further enhance signal diversification. However, receptor specificity of NRG2s appears to be shared with that of NRG1s (7, 9, 12). This observation implies an overlap of signaling pathways by the two NRG families and possible functional redundancy. We aimed at this possibility by making use of synthetic and recombinant forms of NRG2 and NRG1 (α and β isoforms of each), respectively, and a series of interleukin 3 (IL-3)-dependent cell lines expressing defined combinations of ErbB proteins. Our results reveal significant differences between the two isoforms of NRG2. Moreover, each of the four NRG isoforms is distinct in terms of its ErbB specificity. For example, NRG2-α emerges as the broadest specificity factor, whereas the ranges of specificities of NRG2-β and NRG1-α are relatively narrow. Taken together, these results support the notion that the multiple ErbB ligands, through differences in affinity and in specificity to certain receptor dimers, expand the diversification potential of the ErbB signaling module.

MATERIALS AND METHODS

Materials and antibodies. EGF was purchased from Sigma (St. Louis, Mo.), and recombinant NDF-α and NDF-β preparations (EGF-like domains) were from Amgen (Thousand Oaks, Calif.). Radioactive materials were from Amersham (Buckinghamshire, United Kingdom). Iodogen and bis(sulfosuccinimidyl) suberate (BS^3) were from Pierce. Monoclonal antibodies to ErbB proteins (14, 32) were used for immunoprecipitation. A monoclonal antiphosphotyrosine antibody (PY-20; Santa Cruz Biotechnology) was used for Western blot analysis. A murine monoclonal antibody to an active form of MAPK (doubly phosphorylated on both threonine and tyrosine residues of the TEY motif) has been described previously (66). The composition of the buffered solutions has been described previously (62).

Peptide synthesis. NRG2 isoforms were synthesized on an Applied Biosystems (ABI) 430A peptide synthesizer with standard *tert*-butyloxycarbonyl (*t*-Boc) chemistry protocols as provided (version 1.40; *N*-methylpyrrolidone–hydroxybenztriazole). Only the EGF-like domains of NRG2-α and NRG2-β (7, 9, 12) were synthesized. Acetic anhydride capping was employed after each activated ester coupling. The peptides were assembled on phenylacetamidomethyl polystyrene resin by using standard side chain protection, except for the use of *t*-Boc-Glu(*O*-cyclohexyl) and *t*-Boc-Asp(*O*-cyclohexyl). The peptides were deprotected by using the low-high hydrofluoric acid (HF) method (59). In each case, the crude HF product was purified by reverse-phase high-performance liquid chromatography (HPLC) (C_{18} Vydac; 22 by 250 mm), diluted without drying in folding buffer (1 M urea, 100 mM Tris [pH 8.0], 1.5 mM oxidized glutathione, 0.75 mM reduced glutathione, 10 mM methionine), and stirred for 48 h at 4°C. Folded, fully oxidized peptides were purified from the folding mixture by reverse-phase HPLC and characterized by electrospray mass spectroscopy. Peptide quantities were determined by amino acid analysis. Disulfide bonding was analyzed in the following manner. First, the peptide was cleaved with cyanogen bromide (CNBr), which opened up the peptide for further digestion. After removal of CNBr, the peptide was sequentially digested with proteolytic enzymes in order to obtain cleavage between the cysteines. Samples were analyzed by capillary liquid chromatography coupled with electrospray ionization mass spectrometry. The disulfide bonding pattern was determined by using the molecular weights of the fragmented peptides and was shown to be the expected C-1–C-3, C-2–C-4, and C-5–C-6.

Cell lines. The establishment of a series of IL-3-dependent 32D myeloid cells expressing all combinations of ErbB-1, ErbB-2, and ErbB-3 has been described previously (49). To generate an ErbB-4-expressing derivative of 32D cells, we used a long terminal repeat (LTR)-erbB-4 expression vector that was electroporated into 32D cells as described previously (47). Cell lines coexpressing ErbB-2 or ErbB-3, together with ErbB-4, were established by transfection of the pLXSHD retroviral vector (57) directing ErbB-4 expression into ErbB-2- or ErbB-3-expressing cells (D2 and D3 cell lines, respectively) by electroporation (BioRad Genepulser set at 400 V and 250 μF). After a 24-h-long recovery, cells were selected for 4 to 5 weeks in medium containing histidinol (0.4 mg/ml; Boehringer). Clones expressing the two receptors were identified by Western blotting and isolated by limiting dilution. Due to differences in promoter potency, the selected cell line that singly expresses ErbB-4 (D4 cells) contained approximately 10- to 12-fold more ErbB-4 than cell lines expressing the combinations of ErbB-4 with ErbB-2 (D24 cells) or with ErbB-3 (D34 cells).

Radiolabeling of ligands, covalent cross-linking, and ligand binding analyses. Growth factors were labeled with Iodogen (Pierce) as described previously (31). The range of specific activity varied between 2×10^5 cpm/ng (NRG2-α) and 3×10^5 cpm/ng (NRG1-β and NRG2-β). For covalent cross-linking analysis, cells (10^7) were incubated on ice for 1.5 h with either ^{125}I-NRG2-α or ^{125}I-NRG2-β (each at 250 ng/ml). The chemical cross-linking reagent BS^3 was then added (1 mM), and after 1.5 h on ice, cells were pelleted and solubilized in solubilization buffer. For ligand displacement analyses, 10^6 cells were washed once with binding buffer and then incubated for 2 h at 4°C with radiolabeled NRG1-β (5 ng/ml) and various concentrations of an unlabeled ligand, as indicated, in a final volume of 0.2 ml. Nonspecific binding was determined in the presence of a 100-fold molar excess of the unlabeled ligand. To terminate ligand binding, each reaction tube was washed once with 0.5 ml of binding buffer and loaded on top of a 0.7-ml cushion of bovine serum. The tubes were spun (12,000 × *g*, 2 min) in order to remove the unbound ligand.

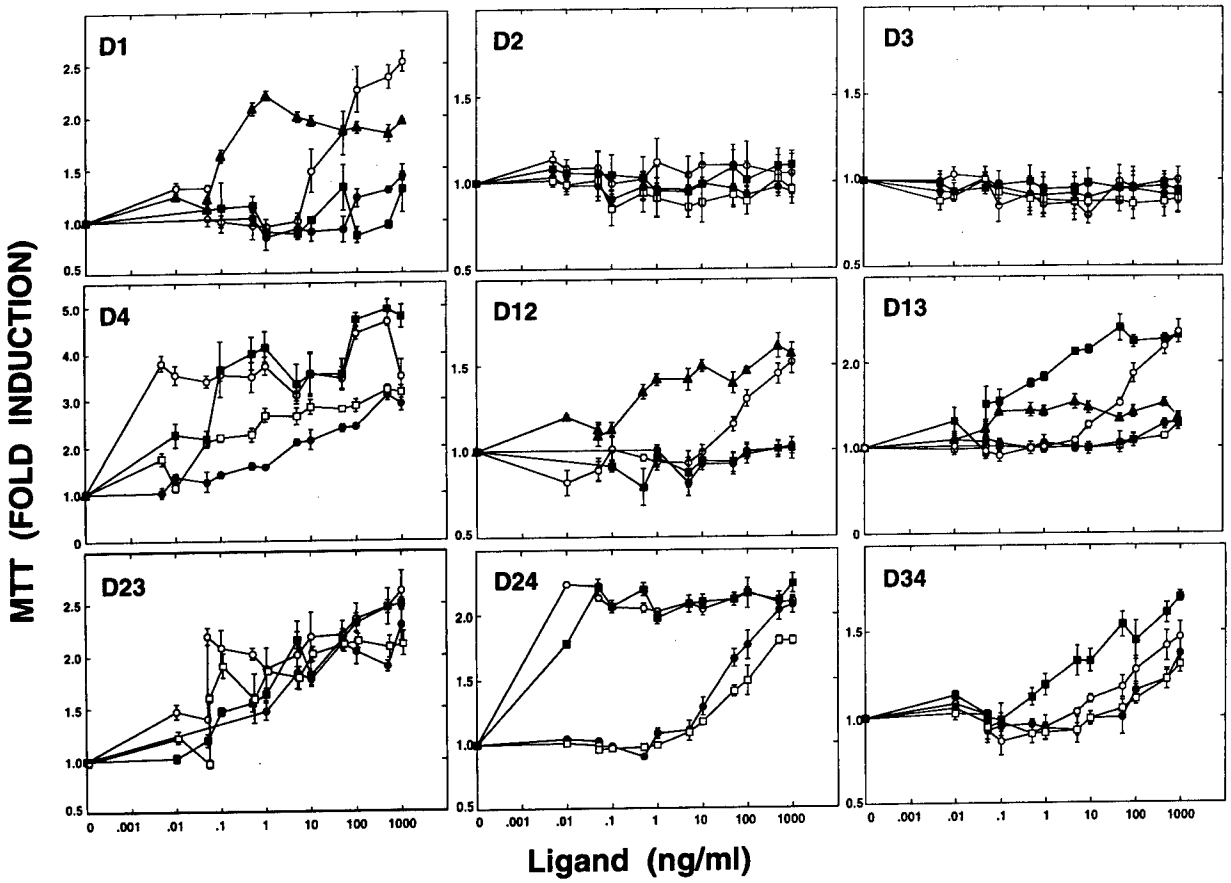


FIG. 2. Proliferative responses of ErbB-expressing derivatives of 32D cells to the four major NRG isoforms. The indicated sublines of 32D cells were tested for cell proliferation by using the MTT assay. Cells were deprived of serum factors and IL-3 and then plated at a density of 5×10^5 cells/ml in media containing serial dilutions of EGF (closed triangles), NRG1-α (open squares), NRG1-β (solid squares), NRG2-α (open circles), or NRG2-β (solid circles). The MTT assay was performed 24 h later. Results are presented as fold induction over the control untreated cells and are the mean ± standard deviation of four determinations. Each experiment was repeated at least twice. Note that no responses were observed with cells expressing either ErbB-2 or ErbB-3 alone, but these cell derivatives retained a response to IL-3.

Lysate preparation, immunoprecipitation, and Western blotting. For analysis of total cell lysates, gel sample buffer was added directly to cell monolayers or suspensions. For other experiments, solubilization buffer was added to cells on ice. Cells were scraped with a rubber policeman into 1 ml of buffer, transferred to microtubes, mixed harshly, and centrifuged (10,000 × *g*, 10 min at 4°C). Rabbit antibodies were directly coupled to protein A-Sepharose beads while shaking for 20 min. Mouse antibodies were first coupled to rabbit anti-mouse IgG and then to protein A-Sepharose beads. The proteins in the lysate supernatants were immunoprecipitated with aliquots of the protein A-Sepharose-antibody complex for 1 h at 4°C. Immunoprecipitates were then washed three times with 20 mM HEPES buffered at pH 7.5–150 mM NaCl–0.1% Triton X-100–10% glycerol (HNTG; 1 ml each wash) prior to being heated (5 min at 95°C) in gel sample buffer. Samples were resolved by gel electrophoresis through 7.5% acrylamide gels and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked for 2 h in TBST buffer (0.02 Tris-HCl buffered at pH 7.5, 0.15 M NaCl, and 0.05% Tween 20) containing 1% milk and blotted with 1 μg of primary antibodies per ml for 2 h, followed by blotting with 0.5 μg of secondary antibody per ml linked to horseradish peroxidase. Immunoreactive bands were detected with an enhanced chemiluminescence reagent (Amersham Corp.).

Cell proliferation assays. Cells were washed free of IL-3, resuspended in RPMI 1640 medium at 5×10^5 cells/ml, and treated without or with growth factors (at 100 ng/ml, unless otherwise indicated) or IL-3 (1:1,000 dilution of conditioned medium). Cell survival was determined by using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl] tetrazolium bromide (MTT) assay as previously described (49). MTT (0.05 mg/ml) was incubated with the cells analyzed for 2 h at 37°C. Living cells can transform the tetrazolium ring into dark-blue formazan crystals that can be quantified by reading the optical density at 540 to 630 nm after lysis of the cells with acidic isopropanol (43).

Cellular differentiation assays. AU-565 human mammary cancer cells were plated in chamber slides (Lab-Tek) and then incubated for 4 days in the absence or presence of ligands (50 ng/ml). Cells were stained with either Oil red O, to visualize neutral lipids, or with a monoclonal antibody to intercellular adhesion molecule 1 (ICAM-1) (Becton Dickinson) as previously described (2). Antibody visualization was performed by using a second incubation with a biotinylated rabbit anti-mouse IgG followed by an alkaline phosphatase-conjugated streptavidin and a red chromogen (Advanced Cellular Diagnostics, Elmhurst, Ill.).

Model building for structure predictions. An initial model for NRG1-β was built in analogy to the structure of human NDF (heregulin) (29) by using coordinates available from the Protein Data Bank (entry 1HRE) and the program Homology (MSI/Biosym, San Diego, Calif.). The coordinates of mouse EGF were similarly obtained from the database (entry 1EPI). The initial model was energy minimized with constraints on Cα positions. The electrostatic potential was computed with the program Delphi (MSI/Biosym package), as has been previously described (22).

RESULTS

NRG isoforms transmit biological signals through distinct receptor combinations. While NRG1-β induces proliferation of many cell types, the factor promotes differentiation of certain mammary cell lines (2, 16, 44). Examination of the two NRG2 isoforms on AU-565 breast cancer cells indicated that both isoforms, like NRG1-β, can promote extensive morphological alterations, induce the appearance of vesicles containing neutral lipids, and up-regulate ICAM-1 (Fig. 1). These differentiation characteristics were shared with the other isoform of NRG1, NRG1-α, but its potency was significantly lower than that of the higher-affinity isoform, NRG1-β (data not shown). Likewise, dose-response analyses of the two NRG2 isoforms revealed that the α isoform was more active

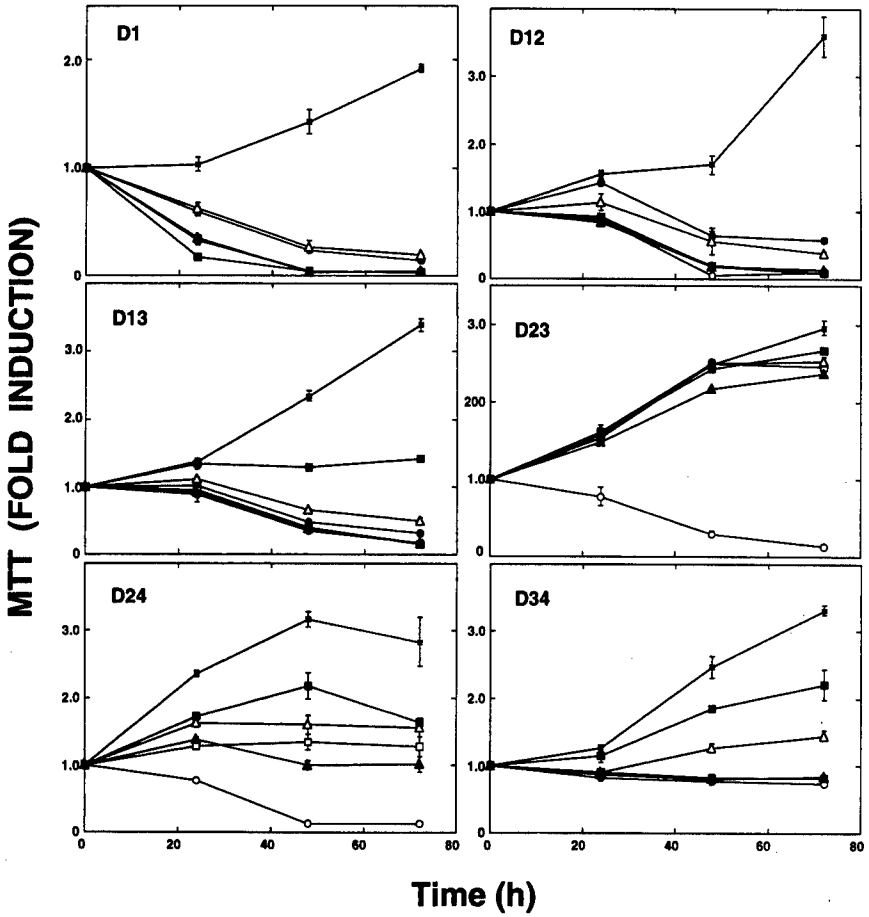


FIG. 3. Ligand-dependent survival of ErbB-expressing 32D cells in the absence of IL-3. The indicated sublines of 32D cells were incubated for various time intervals at a density of 5×10^5 cells/ml in the absence of IL-3 (open circles) or with one of the following ligands, each at a concentration of 100 ng/ml: EGF (solid circles), NRG1-α (open squares), NRG1-β (solid squares), NRG2-α (open triangle), or NRG2-β (solid triangle). For control, cells were incubated with medium conditioned by IL-3-producing cells (crosses). The extent of cell proliferation was determined daily by using the colorimetric MTT assay. The data presented are the mean ± standard deviation of six determinations. The experiment was repeated twice with similar results.

than the β isoform of this family. For example, at a low concentration of NRG2-α (1 ng/ml), approximately 40% of treated cells displayed lipid vesicles, but a similar concentration of NRG2-β was practically inactive (20% positive cells). Taken together with the observation that NRG2-α can stimulate phosphorylation of ErbB-3 and ErbB-4 (7, 9, 12), the results presented in Fig. 1 suggested functional redundancy of the two NRG families.

To directly address this possibility, we performed comparative analysis of receptor specificity of the four NRG isoforms. An extended series of IL-3-dependent 32D myeloid cells that express individual ErbB receptors or their combinations (49) was used in conjunction with the MTT cell proliferation assay. These cells offer the advantage of receptor analysis in the absence of cross talk, because parental 32D cells express no known ErbB molecule. We have previously shown that the MTT assay reflects DNA synthesis and cell cycle progression in this particular cell system (48, 49). Out of the single ErbB-expressing cells, those expressing ErbB-2 alone (denoted D2 cells), as well as cells expressing the kinase-defective ErbB-3 protein alone (D3 cells), responded to no NRG isoform (Fig. 2). In contrast, D4 cells, which express ErbB-4 at relatively high levels, underwent enhanced proliferation in response to all four NRG isoforms (Fig. 2). Surprisingly, cells singly expressing ErbB-1 (D1 cells) responded to NRG2-α, but they responded only weakly to very high concentrations of NRG2-β (Fig. 2). None of the two NRG1 isoforms was active on the ErbB-1-expressing 32D cells at concentrations as high as 100 ng/ml. In comparison with EGF, whose activity on D1 cells was detectable with as low a concentration as 0.1 ng/ml, the concentration of NRG2-α needed to elicit a similar response was at least 10-fold higher. While part of this discrepancy may be due to incomplete refolding of the synthetic NRG2 molecules we used, it is worthwhile noting that the NRG2-α-mediated effect exceeded, at high concentrations, the maximal response to EGF. In addition, long-term survival assays, which were performed with a single high dose of ligand, indicated that NRG2-α acted at least as efficiently as EGF in extending cell survival in the absence of IL-3 (Fig. 3). These observations, together with the specificity to NRG2-α, appear to attribute physiological relevance to the interaction between ErbB-1 and NRG2-α

Examination of cell lines expressing various pairs of ErbB proteins revealed an overall isoform-specific pattern of dimer specificity: with all receptor combinations, NRG2-α was more potent than NRG2-β, whereas NRG1-β was superior to NRG1-α on cells expressing either ErbB-3 or ErbB-4 (Fig. 2 and 3). The relative potency, however, of the two more active NRG isoforms, NRG1-β and NRG2-α, displayed dimer dependency. For example, cells expressing a combination of

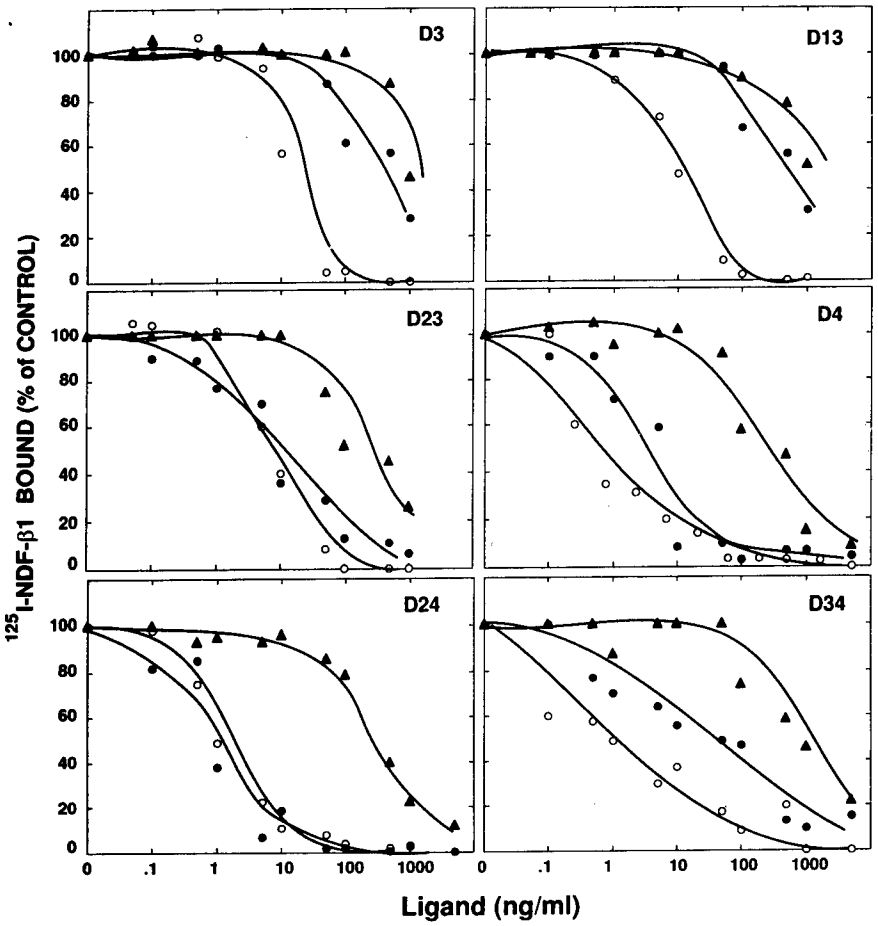


FIG. 4. Binding of type 2 neuregulins to specific ErbB proteins. Displacement analyses of radiolabeled NRG1-β were performed with the indicated derivatives of 32D cells. Cells (10^6) were incubated for 2 h at 4°C with the radiolabeled ligand (1 ng/ml) in the presence of increasing concentrations of an unlabeled NRG2-α (closed circles), NRG2-β (closed triangles), or NRG1-β (open circles). To remove unbound ligands, cells were sedimented (12,000 × *g*, 2 min) through a cushion of calf serum at the end of the experiment, and their radioactivity was determined. Nonspecific binding of NRG1-β was determined in the presence of 100-fold excess of the unlabeled ligand. Each data point represents the mean (less than 10% variation) of two determinations.

ErbB-1 and ErbB-3 (D13 cells) were most efficiently stimulated by NRG1-β, which also acted as a potent survival factor for these cells (Fig. 3). D13 cells, however, responded to NRG2-α better than to EGF, and the two other NRG isoforms (NRG1-α and NRG2-β) were practically inactive (Fig. 2 and 3). A cooperative effect of ErbB-2 on binding (45, 55, 61) and cellular responses (23, 30, 49) to NRG1 has been previously described. This effect extends to NRG2 isoforms: coexpression of ErbB-2 and ErbB-3 sensitized cells to low concentrations of both types of NRG2 isoforms, and it also enhanced their potency to a level comparable to that of IL-3 (Fig. 2 and 3). In addition, the combination of ErbB-2 with ErbB-4 displayed remarkable sensitivity to NRG1-β and to NRG2-α (Fig. 2). For example, D34 cells that express ErbB-4 at the same level of D24 cells, but at least 10-fold lower than D4 cells, displayed significantly lower sensitivity to the more potent NRG isoforms (Fig. 2). In conclusion, the four NRG isoforms are distinct in their range of receptor specificity, and they collectively recognize all stimulatable receptor combinations. Consequently, the resulting cellular responses display a graded pattern ranging from weak to potent mitogenicity (Fig. 2) and survival (Fig. 3).

Cooperative and isoform-specific recognition of ErbB proteins. Because previous comparison of the two NRG1 isoforms revealed remarkable quantitative (60) and qualitative differences (48), it was interesting to analyze binding specificities and relative affinities of the two NRG2 isoforms and correlate them with the observed differences in biological response. First, we compared the capacity of each NRG2 isoform to displace a cell-bound radioactive NRG1-β. In line with the mitogenic superiority of the α isoform of NRG2, this type of isoform acted more efficiently than NRG2-β in the ligand displacement assay, on cells expressing all types of receptor combinations (Fig. 4). Similar to NRG1 isoforms, whose higher-affinity receptor is ErbB-4 (60), both types of NRG2s appear to bind to ErbB-4 with higher affinity than to the other receptor, ErbB-3 (compare D3 and D4 panels in Fig. 4). In agreement with the cooperative effect of ErbB-2, which was observed in both the cell proliferation assay and in the survival assay, coexpression of ErbB-2 together with ErbB-3 led to a 50-fold enhancement of NRG2-α affinity (Fig. 4). In fact, coexpression of ErbB-2 with ErbB-4 resulted in a greater affinity to NRG2-α than to NRG1-β, but the ErbB-4-ErbB-3 combination (D34 cells, Fig. 4) was not cooperative in terms of apparent ligand affinity.

Due to the relatively low affinity of NRG2 isoforms to ErbB-1, displacement of radiolabeled EGF from this receptor was inefficient (data not shown). Therefore, we used radiola-

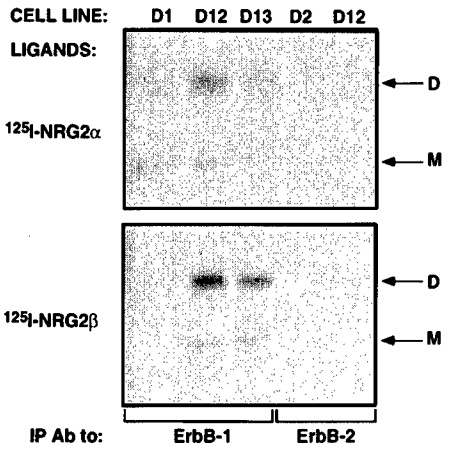


FIG. 5. Covalent cross-linking of radiolabeled NRG2 isoforms to ErbB-1-expressing cells. The indicated cells (10^7 cells per lane) expressing various ErbB proteins, including control cells expressing ErbB-2 alone (D2 cells), were incubated with either ^{125}I-NRG2-α or with ^{125}I-NRG2-β (each at 250 ng/ml). Following 90 min at 4°C, the covalent cross-linking reagent BS^3 was added (1 mM, final concentration), and cell lysates were prepared after an additional 1.5 h of incubation. Affinity-labeled ErbB-1, ErbB-2, or ErbB-3 was immunoprecipitated by using specific mouse monoclonal antibodies, and the complexes were resolved by gel electrophoresis and autoradiography. Arrows mark the locations of monomeric (M) and dimeric (D) receptor complexes.

beled derivatives of NRG2 molecules and covalent cross-linking analysis to assay binding to ErbB-1 (Fig. 5). Evidently, both types of NRG2 molecules, when radiolabeled, displayed specific binding to monomers and dimers of ErbB-1. Presumably, NRG2-β binds to ErbB-1 with an affinity that is too low to allow mitogenicity (Fig. 2), but the procedure of covalent cross-linking makes this weak recognition detectable. Consistent with a cooperative effect, ErbB-2 specifically enhanced labeling of the dimeric form in D12 cells, although immunoprecipitation analysis implied that by itself ErbB-2 underwent only limited labeling by the radioactive ligand (Fig. 5). Specificity of labeling by NRG2s was evident by the absence of covalent cross-linking of ErbB-2, when singly expressed (D2 cells, Fig. 5), and by displacement with unlabeled EGF (data not shown). Taken together with the results of the displacement assay, our binding data support a model of isoform-specific pattern of receptor recognition.

Receptor phosphorylation and MAPK activation display distinct ligand-specific patterns. The remarkable differences we observed when comparing the actions of NRG isoforms in respect to cell proliferation and survival suggested that the distinct pairs of ligands and dimeric receptors differ in their signaling potencies. Indeed, comparisons of receptor phosphorylation on tyrosine residues were in line with the results obtained in the biological tests (Fig. 6). Whereas EGF stimulated extensive tyrosine phosphorylation of its receptor in D1 cells, the less-potent ligand, NRG2-α, induced a smaller effect, and the nonmitogenic ligand isoforms (NRG1s and NRG2-β) failed to stimulate tyrosine phosphorylation in these cells at a concentration of 100 ng/ml (Fig. 6A). In D13 cells, the most potent NRG isoform, NRG1-β, elicited higher tyrosine phosphorylation than the less potent NRG2-α isoform, while EGF was as effective as NRG1-β (Fig. 6A), probably because ErbB-1 expression exceeded the level of ErbB-3 in these cells. Examination of cells expressing various combinations of ErbB-2, ErbB-3, and ErbB-4 led to a similar conclusion, namely, that the extent of tyrosine phosphorylation of high-molecular-weight proteins, most likely activated ErbBs, correlated with the relative mitogenic potency of NRG isoforms (Fig. 6B).

Because MAPKs are stimulated by all ligand-activated combinations of ErbB proteins (23, 30, 49), and they can integrate incoming signals (38, 54), we attempted to correlate the mitogenic potencies of NRGs with patterns of MAPK activation. Toward this end, we made use of a murine monoclonal antibody that specifically recognizes the active, doubly phosphorylated form of the ERK1 and ERK2 MAPKs (66). Immunoblotting of whole-cell lysates of D1 cells with this antibody revealed differences between the kinetics of MAPK activation by EGF and NRG2-α. In both cases, a delay of MAPK activation, compared to receptor phosphorylation, was observed, but receptor activation was more sustained with the more potent mitogen, EGF (Fig. 7A). Remarkably, the higher-molecular-weight form of MAPK, p44/ERK1, underwent activation only in response to EGF, and its kinetics were delayed. D4 cells, whose mitogenic responsiveness to NRGs was relatively high (Fig. 2), displayed relatively sustained and potent stimulation of MAPK (Fig. 7A), probably because these cells express approximately 10-fold more receptors than other derivative lines. Although the mitogenic action of the more potent NRGs, NRG1-β and NRG2-α, were comparable, (D4 panels in Fig. 2), MAPK activation was more prolonged in the case of NRG1-β, in agreement with the higher binding affinity of this ligand to ErbB-4 (Fig. 4). In D4, as well as in D23 cells, in which stimulation by NRGs was as potent as with IL-3 (Fig. 3), treatment with either NRG1-β or NRG2-α led to a robust and concomitant stimulation of both ERK1 and ERK2. Yet another pattern was shared by the two NRGs in D24 cells: both ERK isoforms were stimulated at the same early time point (1 min), but they, along with the receptors, displayed a relatively long decay (up to 120 min).

Analysis of MAPK activation by the relatively weak NRG isoforms, namely, NRG1-α and NRG2-β, extended the correlation with mitogenic activity and further supported the cooperative effect of ErbB-2 (Fig. 7B). Consistent with their weak or no mitogenic effect on D1 and D13 cells, the two isoforms induced practically no activation of MAPK in the two cell lines, but EGF was active in this assay. NRG1-α was more potent than NRG2-β on D4 cells, consistent with its higher mitoge-

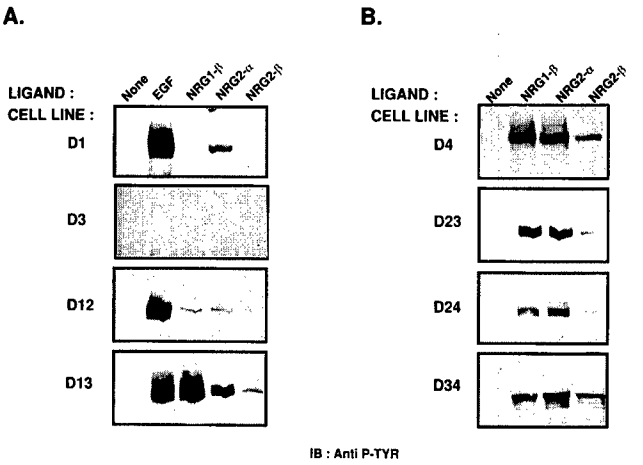
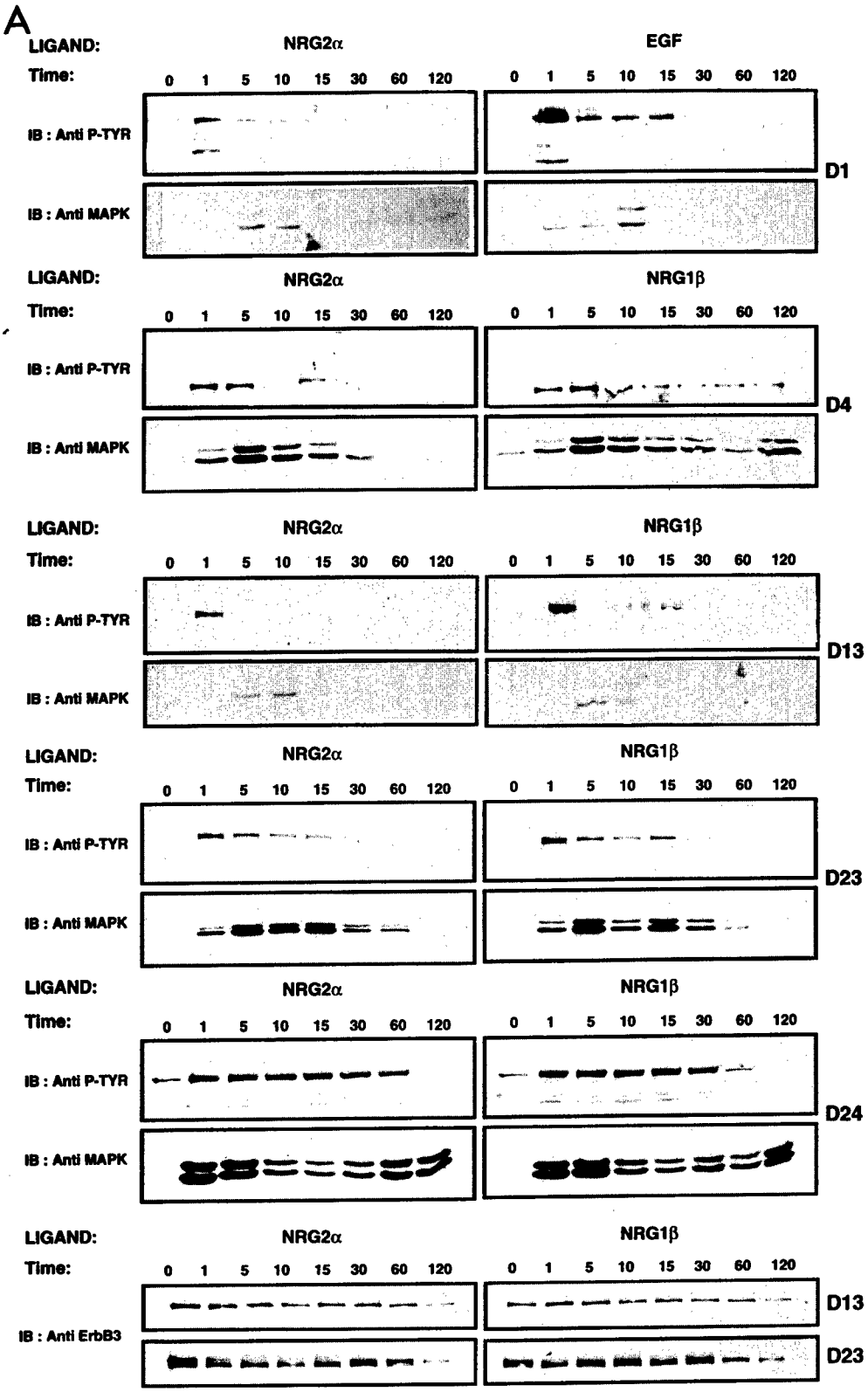


FIG. 6. NRG2-induced tyrosine phosphorylation of ErbB proteins. The indicated cell lines were incubated for 1 min at 37°C with either EGF, NRG1-α, NRG1-β, NRG2-α, or NRG2-β, each at 100 ng/ml. Control cultures were incubated with no added factor (None). Whole-cell lysates were then prepared, cleared from cell debris, and subjected to an immunoblot analysis with the PY-20 antiphosphotyrosine antibody. The regions of the gels corresponding to apparent molecular masses of 150 to 200 kDa are shown.



nicity for these cells. Finally, coexpression of ErbB-2, with either ErbB-3 or ErbB-4, significantly enhanced MAPK activation by the two relatively weak isoforms of NRG (Fig. 7B, D23 and D24). Taken together, the results presented in Fig. 7 indicate that the four isoforms of NRG, when acting through the four ErbB proteins, are able to set the MAPK pathway at different levels of activation, thus offering a basis for differences in biological potencies.

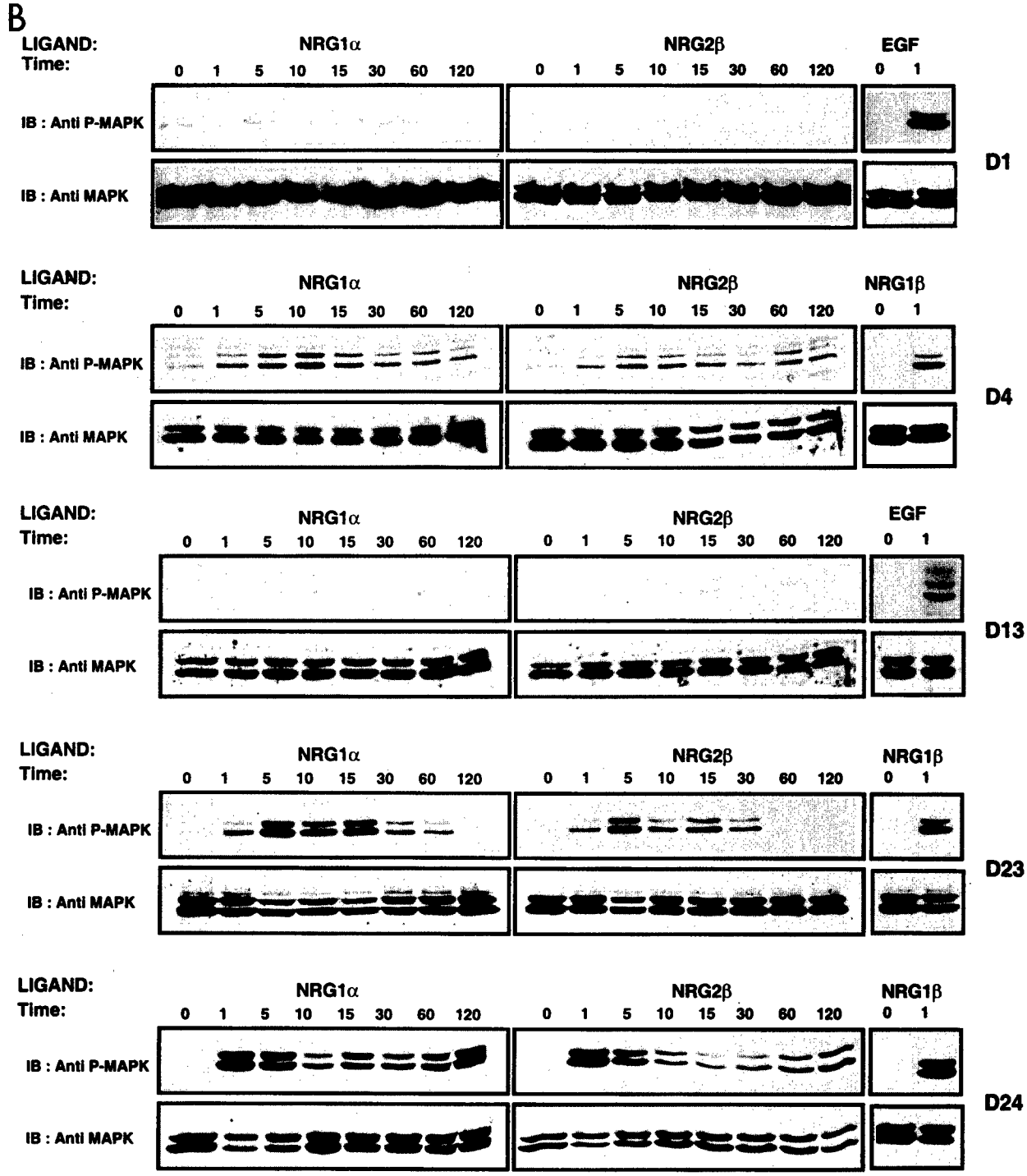


FIG. 7. Kinetics of receptor phosphorylation and MAPK activation by NRGs. The indicated derivatives of 32D cells were incubated for various time intervals (indicated in minutes) with growth factors (each at 100 ng/ml). All four isoforms of NRG1 and NRG2, along with EGF, were tested. Results obtained with the two more potent isoforms, NRG1-β and NRG2-α, are shown in panel A, and those obtained with the weaker factors, NRG1-α and NRG2-β, are shown in panel B. At the end of the incubation, period, whole-cell lysates were prepared, cleared, and subjected to immunoblotting (IB) with either an antibody to phosphotyrosine (P-TYR) or with an antibody specific to the active doubly phosphorylated form of MAPK (66). Immunoblotting of whole-cell lysates with antibodies to ErbB-3 (A, bottom panels) or to the MAPK (B) were used to compare protein loading. Signal detection was performed by using a chemiluminescence kit.

DISCUSSION

Utilizing synthetic versions of the two newly reported NRG2 isoforms on a cellular system whose ErbB repertoire is defined, we identified a network of ligand-receptor interactions that is distinct from the one employed by NRG1 isoforms. Nevertheless, these two networks, which are schematically presented in Fig. 8, are partly overlapping and share several characteristics, including recruitment of ErbB-2 and its cooperative action, lack of interaction with homodimers of ErbB-2, and pairing of a relatively high-affinity ligand, whose range of receptors is

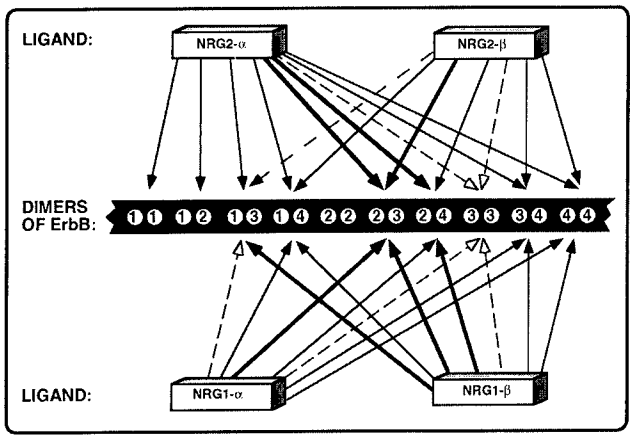


FIG. 8. Summary of ligand-receptor interactions within the NRG-ErbB signaling network. The horizontal gray bar represents the plasma membrane, and the various receptor combinations are shown schematically as circles. Specific ErbB proteins are identified by their numbers. The four major NRG isoforms are shown, and their strengths of signaling, as revealed by using the IL-3-dependent series of cell lines, are shown by arrows. Bold arrows indicate potent proliferative responses at low ligand concentrations (1 ng/ml or less). Note that no NRG isoform is able to activate the ErbB-3 homodimer (broken arrows), although all isoforms bind to this dimer. Likewise, NRG1-α cannot activate the ErbB-1/ErbB-3 heterodimer (48). In addition, no ligand binds to the ErbB-2 homodimer, but heterodimers of this protein with ErbB-3 or with ErbB-4 are relatively potent combinations. The information regarding the ErbB-1/ErbB-4 heterodimer was derived from Chinese hamster ovary cells overexpressing the two proteins (62). All other receptor combinations were examined in 32D cell derivatives.

broad (i.e., NRG1-β and NRG2-α), with a low-affinity ligand that binds to a relatively small set of dimeric ErbB combinations (NRG1-α and NRG2-β). Because spatial and temporal patterns of NRG1 expression are different from those exhibited by the more restricted NRG2 family (7, 9, 12), and the two isoforms of each family are expected to have yet their own distinct patterns (13, 40), the observed differences in receptor specificity are expected to increase functional diversity. Indeed, initial in vitro analyses of NRG1 and NRG2 revealed both quantitative and qualitative differences in activation of epithelial, muscle, and Schwann cells (6, 7).

It is worth noting that the structural difference between the α and β isoforms of NRG1, as well as NRG2 (Fig. 9A), is confined to the third loop of the EGF-like domain (loop C) and to the adjacent C terminus. This domain, however, is not the major site of structural variation, because the membrane proximal region, which connects the EGF-like domain of NRGs with the transmembrane stretch of proNRG molecules, displays broader variation (7, 27, 64). Whereas the juxtamembrane variation affects the rate of precursor processing, the more proximal heterogeneity, which represents alternative usage of one of two exons encoding the C-terminal loop of the EGF-like domain (7), critically influences receptor binding affinity (Fig. 4). The quantitative difference in affinity between NRG2 isoforms may translate into a qualitative one, since the analogous alteration in NRG1 dictates the differential ability of NRG1 isoforms to recruit ErbB-1 into a dimer with ErbB-3 (48). Likewise, the differences in receptor recognition displayed by the two direct ligands of ErbB-1, EGF and TGFα, are also due to a specific C-terminal sequence (34). In contrast, construction of hybrids between NRG1 and EGF revealed that the N terminus, rather than the C terminus, confers to NRG1 the ability to bind to its primary receptor (3). These observations can be explained by a model that attributes bivalence to NRG molecules (61). Accordingly, the N-terminal part of the molecule allows high-affinity binding to a primary receptor, whereas the variant C-terminally located site confers an ability to recruit a secondary receptor. A bivalency model may apply also to EGF, because this ligand undergoes covalent cross-linking to different portions of ErbB-1, depending on whether cross-linking is mediated by the N or C terminus of EGF (58). In terms of bivalent ligand-receptor interactions, the broader and more potent signaling by NRG2-α is probably due to the C-terminally located binding site, whose affinity and range of ErbB specificity are larger than those of the corresponding site of NRG2-β.

Strikingly, all EGF-like ligands of ErbB proteins share very similar structures in their folded forms (29). This is dictated by the three-loop secondary structure and by a bilobular β structure that is held by hydrogen bonds. Interestingly, the middle loop of NRG1 (loop B, Cys2-Cys4) is longer by three amino acids than that of NRG2 (Fig. 9A). A similarly shorter loop exists in all ErbB-1-specific ligands, including EGF and TGFα. This structural feature may contribute to the ability of NRG2-α, but not NRG1s, to activate ErbB-1 in the absence of other ErbBs (Fig. 2 and 3). An alternative explanation is derived from the predicted folded structure of NRG2-α (Fig. 9B): although the compact structure of this ligand is in general similar to that of EGF and NRG1-β, the expected distribution of surface charges, especially in the C terminus, is more similar to that of EGF than to the practically neutral C tail of NRG1-β. In light of these considerations, it is worthwhile to address the question of why previous analyses did not detect interaction between NRG2 and ErbB-1 (7, 9, 12). Both Chang et al. (12) and Carraway et al. (9) used only the less potent isoform, NRG2-β, which is unable to stimulate ErbB-1 under normal conditions (Fig. 6A). Nevertheless, Carraway et al. (9) observed NRG2-induced ErbB-1 phosphorylation in MDA-MB468 cells, which express extremely high levels of ErbB-1. Possibly, ErbB-1 overexpression and the relatively high concentrations of recombinant NRG2-β used by these investigators enabled them to detect the weak interaction of NRG2-β with ErbB-1. Although, Busfield et al. used the higher-affinity ligand, NRG2-α (DON-1), none of their assays was aimed at detecting ErbB-1 activation. Apart from the interaction of NRG2-α with ErbB-1, our results are in full agreement with those of the three previous reports on NRG2. In fact, the observation that NRG1-β is more potent than NRG2-β in induction of epithelial cell flattening (12) and the evidence for better mitogenic response of mammary cells to NRG2-α than to NRG1-α (7) are consistent with the network we observed by using engineered myeloid cells (Fig. 8). Also consistent is the superiority of NRG1-β over NRG1-α in up-regulation of the acetylcholine receptor of chick muscle cells (7), but the complete inactivity of NRG2-α in this system may be attributed to a species barrier.

Our conclusion that each NRG isoform acts through a distinct set of dimeric receptors further extends the already large diversification potential of the ErbB signaling network (1). Three levels of diversity generation may be defined: In addition to the 10 dimeric receptor complexes, whose formation is ligand dependent and hierarchical (62), diversity is generated at the level of the multiple ligands, and more complexity is contributed by the many cytoplasmic signaling proteins that are recruited by each dimeric receptor complex. The ligand level exhibits remarkable diversity: Each ligand appears to differ from the others by its unique receptor specificity. Examples are betacellulin and the heparin-binding EGF-like growth factor, which bind to ErbB-4, in addition to ErbB-1 (18, 51) and EGF, an ErbB-1 ligand capable of activating the ErbB-2/ErbB-3 heterodimers at high concentrations (49a). Surpris-

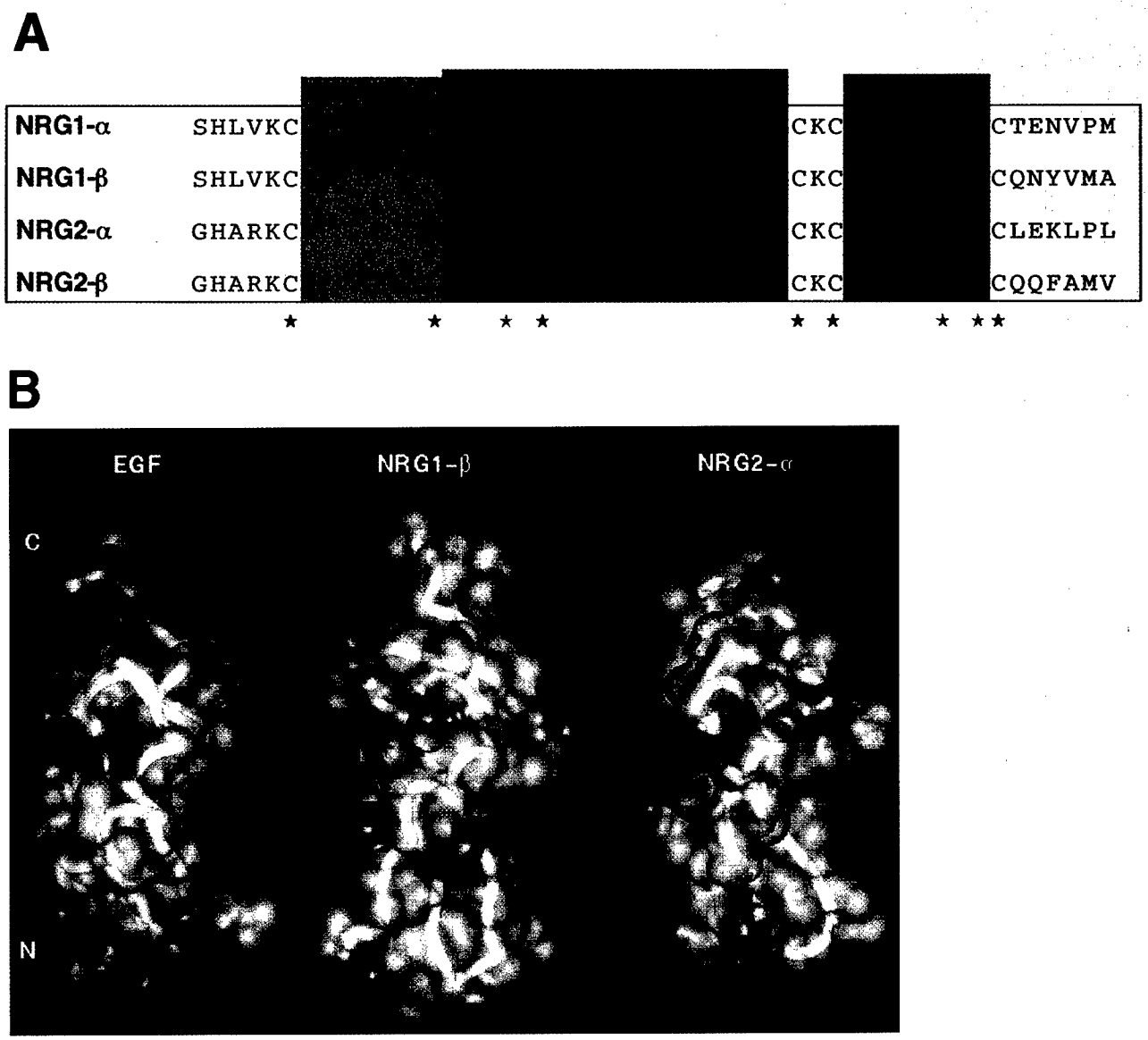


FIG. 9. Comparison of amino acid sequences and electrostatic potentials of three EGF-like ligands. (A) Alignment of amino acid sequences of the EGF-like domains of NRG1 and NRG2 isoforms. The three disulfide loops (A through C) are indicated, including the region shared by loops A and B (J region). Asterisks mark the canonical residues of the EGF-like family of ligands. (B) The figure depicts the solvent-accessible surfaces of the EGF-like domains of the molecules mouse EGF, rat NRG1-β, and rat NRG2-α. The molecules are colored according to their electrostatic potential: red for negative potential and blue for positive potential. Neutral areas are shown in white. The surfaces are transparent to show ribbon diagrams of the molecules (yellow). The locations of the N and C termini are indicated. Note the relatively extended structure of NRG-1 and its neutral C terminus. In contrast, the C termini of both EGF and NRG2-α are charged. Note that the N termini of the two types of NRG, a region that dictates high-affinity binding to ErbB-3 (3, 61), share a positive surface potential.

ingly, the third layer of signal diversification, namely, the effector molecules, displays only limited variation. Although each ErbB protein carries a distinct set of potential docking sites for cytoplasmic signaling proteins (10), only a few receptor-specific substrates have been actually identified. These include c-Cbl (36) and phospholipase Cγ (15, 20), which are substrates of ErbB-1 and ErbB-2, but are unable to couple to ErbB-3 and ErbB-4. On the other hand, many signaling proteins, like Shc, Grb-2, and phosphatidylinositol 3′ kinase (20, 50), are shared by the four ErbB molecules. Because we observed different patterns of MAPK activation upon cell stimulation with NRG2 (Fig. 7), and previous reports documented a similar phenomenon with other ligands, namely NRG1s and EGF (23, 30, 49), we raise an alternative mechanism of signal diversification at the effector level. Accordingly, specificity of signaling is due to the variable degree of coupling to the MAPK pathway, rather than to an ErbB dimer-specific substrate(s). Thus, transient and weak activation of MAPK (especially ERK1) characterizes homodimers of ErbB-1, and sustained activation is observed with NRG-stimulated heterodimers of ErbB-2 with either ErbB-3 or with ErbB-4 (Fig. 7). The prolongation effect of ErbB-2 has been previously reported in mammary tumor cells and correlated with the extent of overexpression of this oncogenic protein (30). Conceivably, ErbB-2 prolongs NRG-mediated MAPK activation by its cooperative effect on ligand binding (Fig. 4). Additional factors that may extend MAPK activation are the relatively strong coupling of ErbB-2 to this pathway (5) and the uniquely slow rate of ErbB-2 endocytosis (56). Thus, the network of NRGs and ErbBs is able to translate the strength of ligand-receptor

interactions to different patterns of MAPK activation. This model is consistent with many results obtained in pheochromocytoma cells (PC-12), in which a correlation between the kinetics of MAPK activation and the type of cellular response, either proliferation or differentiation, was established (reviewed in reference 39). Finally, because only one ligand-ErbB pair exists in lower organisms, it is tempting to propose that the network of NRG and ErbB proteins represents a machinery developed throughout evolution for fine tuning of the MAPK pathway. Each of the multiple mammalian ErbB ligands may thus determine a specific setting of the ErbB module and consequently lead to cellular proliferation, survival, or differentiation. When fully active, like in the case of epithelial cells overexpressing ErbB-2 or maintaining NRG autocrine loops (for review, see reference 53), this pathway may contribute to cancer development.

ACKNOWLEDGMENT

This work was supported by a grant from the Department of the Army (grant DAMD 17-97-1-7290).

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THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 273, No. 17, Issue of April 24, pp. 10496–10505, 1998
Printed in U.S.A.

Epiregulin Is a Potent Pan-ErbB Ligand That Preferentially Activates Heterodimeric Receptor Complexes*

(Received for publication, November 13, 1997, and in revised form, February 7, 1998)

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The ErbB signaling network consists of four transmembrane receptor tyrosine kinases and more than a dozen ligands sharing an epidermal growth factor (EGF) motif. The multiplicity of ErbB-specific ligands is incompletely understood in terms of signal specificity because all ErbB molecules signal through partially overlapping pathways. Here we addressed the action of epiregulin, a recently isolated ligand of ErbB-1. By employing a set of factor-dependent cell lines engineered to express individual ErbBs or their combinations, we found that epiregulin is the broadest specificity EGF-like ligand so far characterized: not only does it stimulate homodimers of both ErbB-1 and ErbB-4, it also activates all possible heterodimeric ErbB complexes. Consistent with its relaxed selectivity, epiregulin binds the various receptor combinations with an affinity that is approximately 100-fold lower than the affinity of ligands with more stringent selectivity, including EGF. Nevertheless, epiregulin's action upon most receptor combinations transmits a more potent mitogenic signal than does EGF. This remarkable discrepancy between binding affinity and bioactivity is permitted by a mechanism that prevents receptor down-regulation, and results in a weak, but prolonged, state of receptor activation.

Various biological processes are controlled by intercellular interactions that are mediated by polypeptide growth factors. Examples include embryonic development, neuronal functions, hematopoiesis, and pathological situations, like wound healing and malignant transformation. The mechanism transmitting extracellular signals ultimately starts with binding of the growth factor to a cell surface receptor, that in many cases carries an intrinsic tyrosine kinase activity (1). These receptors fall into several subgroups sharing structural and functional characteristics. Each subgroup of receptors specifically recognizes a family of structurally homologous growth factors. Perhaps the most striking multiplicity of related growth factors is exemplified by the epidermal growth factor (EGF)[1] family of molecules (2). This six cysteine-containing motif of 45–60 amino acids is shared by all members of the family, and it functions as the receptor binding portion of the molecule. Currently there are four known receptors for EGF-like ligands, constituting the ErbB subgroup of receptor tyrosine kinases (also known as HER, or type I receptor tyrosine kinases (3)). Whereas ErbB-1 binds many ligands, including EGF, transforming growth factor α (TGFα), and amphiregulin, both ErbB-3 and ErbB-4 bind to a family of isoforms, collectively known as neuregulins (also called Neu differentiation factors, heregulins, glial growth factors, and acetylcholine receptor inducing activity) (4). A related group of molecules, termed NRG2, binds to the same two receptors (5–7), and a third molecule, NRG3, exclusively binds to ErbB-4 (8). Two other ligands, betacellulin (9), and the heparin-binding EGF-like growth factor (10, 11) bind to both ErbB-1 and ErbB-4. Interestingly, the most oncogenic member of the ErbB family, ErbB-2, binds none of the EGF-like ligands with high affinity. However, recent studies indicate that ErbB-2 functions as a shared low affinity receptor that binds the apparently bivalent EGF-like ligands with low affinity, once they are presented by either one of the high affinity receptors (12).

Despite shared receptor specificity, it is clear that the multiple EGF-like ligands play distinct physiological roles: gene targeting experiments showed that loss of function of ErbB-1 (13–15) more severely impairs embryonic development than inactivation of one of its ligands, TGFα (16). On the other hand, targeting of either neuregulin (17), ErbB-2 (18), or ErbB-4 (19), resulted in the same embryonic cardiac defect, indicating that activation of an ErbB-2/ErbB-4 receptor combination is exclusively mediated by neuregulin in the developing heart. That ligand multiplicity related to tissue-specific expression is suggested by distinct spatial and temporal patterns of expression of the various ligands (reviewed in Ref. 2), and also by experiments with transgenic mice demonstrating tissue selectivity of specific ErbB-1 ligands (20). Part of the physiological selectivity of ligands with shared receptors may be attributed to their domains that flank the EGF-like motif, including the presence of heparin-binding sites, sugars, and specific protein motifs.

In this study we addressed the functional identity of epiregulin, a recently identified ligand of ErbB-1 (21, 22). Like TGFα, this ligand was originally isolated from the medium of transformed fibroblasts, and its transmembrane precursor carries only short sequences that flank the EGF-like motif. Epiregulin expression is relatively restricted; except for macrophages and placenta, other human tissues contain very low or no epiregulin transcripts, but most types of epithelial tumors are characterized by high expression of the growth factor (23). Although

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[1] The abbreviations used are: EGF, epidermal growth factor; CHO, Chinese hamster ovary; IL-3, interleukin 3; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NDF, Neu differentiation factor; TGF-α, transforming growth factor α.

epiregulin competed with EGF on the binding to ErbB-1, it displayed relatively low affinity to ErbB-1-overexpressing cells (21). On the other hand, the factor displayed dual biological function *in vitro*: it stimulated proliferation of fibroblasts, smooth muscle cells, and hepatocytes, but inhibited growth of several tumor-derived epithelial cell lines (21). These observations, and the emerging broader than expected specificity of EGF-like ligands to ErbB proteins (reviewed in Ref. 24), prompted us to analyze the selectivity of epiregulin to ErbB proteins. Here we report that epiregulin is a pan-ErbB ligand that activates all ligand-stimulatable combinations of ErbB proteins with variable efficiency. Strikingly, in a model cellular system, epiregulin more potently activates mitogenesis than does EGF, although the affinity of EGF to ErbB-1 is approximately 100-fold higher. This superiority of epiregulin is independent on the presence of other ErbB proteins, and appears to result from a relatively inefficient mechanism of receptor inactivation.

EXPERIMENTAL PROCEDURES

Materials, Buffers, and Antibodies—A recombinant form of NDF-$\beta 1_{177-246}$ was kindly provided by Amgen (Thousand Oaks, CA). Human recombinant EGF and TGFα were purchased from Sigma. Radioactive materials were purchased from Amersham (Buckinghamshire, United Kingdom). IODO-GEN and BS^3 were from Pierce. A monoclonal antibody to the ErbB-2 protein, mAb L26 (25), was used to stimulate ErbB-2. A monoclonal anti-phosphotyrosine antibody (PY-20, Santa Cruz Biotechnology) was used for Western blot analysis. A mAb to the active form of MAPK (doubly phosphorylated on both tyrosine and threonine residues of the TEY motif) (26) was a gift from Rony Seger. Binding buffer contained Dulbecco's modified Eagle's medium with 0.5% bovine serum albumin and 20 mM HEPES. Solubilization buffer contained 1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM EGTA, 1.5 mM $MgCl_2$, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, aprotinin (0.15 trypsin inhibitor unit/ml), and 10 μg/ml leupeptin.

Peptide Synthesis—Epiregulin was synthesized on an Applied Biosystems (ABI) model 431 peptide synthesizer fortified with UV feedback monitoring at 301 nm, and using Fmoc (9-fluorenylethoxycarbonyl-)-Rink amide AM resin. Only the EGF-like domain of the murine epiregulin (21) was synthesized. The conventional ABI monitor previous peak algorithm was employed up to five times with a cut-off of 3.5% of the first deprotection. A secondary deprotection was performed and followed by double coupling. Acetic anhydride/1-hydroxybenzotriazole capping was utilized at the end of each coupling, followed by washing with 1:1 trifluoroethanol/dichloromethane. The peptide was deprotected and removed from the resin as described (27), with the following modifications: methoxyindole (2%) was added to reagent K, and the reaction time was changed to 3.5 h. Small quantities of the reduced peptides were purified by reverse-phase high performance liquid chromatography and examined by matrix-assisted laser desorption ionization mass spectral analysis. The crude reduced protein was dissolved in a Tris-HCl buffer, pH 6.0, containing guanidium HCl (6 M) and diluted to a concentration of 0.06 mg/ml in methionine-containing buffer (10 mM) that included 1.5 mM cystine, 0.75 mM cysteine, and 100 mM Tris, pH 8.0. The mixture was stirred for 48 h at 4 °C, and the oxidized protein isolated on a C-4 VYDAC 10 micron preparative column (22 × 250 mm) using a 0.1% trifluoroacetic acid/water/acetonitrile gradient. The oxidized protein was lyophilized and characterized by mass spectrometry and amino acid analysis, and shown to be homogeneous. Electrospray mass spectrometry was used to verify the mass of the synthetic peptide.

Cell Lines—MDA-MB453 cells were purchased from the American Type Culture Collection (Rockville, MD). The Chinese hamster ovary (CHO) cell lines expressing various ErbB proteins or their combinations were described previously (28). The establishment of a series of interleukin 3 (IL-3)-dependent 32D myeloid cells expressing all combinations of ErbB-1, ErbB-2, and ErbB-3 has been described (29). To generate an ErbB-4-overexpressing derivative of 32D cells, we used an LTR-*erbB-4* expression vector that was electroporated into 32D cells as described (30). Cell lines co-expressing ErbB-2 or ErbB-3, together with ErbB-4, were established by transfection of the pLXSHD reteroviral vector (31), directing ErbB-4 expression, into ErbB-2- or ErbB-3-expressing cells (D2 and D3, respectively) by using electroporation (Bio-Rad Genepulser, set at 400 volts and 250 millifarad). After a 24-h long recovery, cells were selected for 4–5 weeks in medium containing histidinol (0.4 mg/ml). Clones expressing the two receptors were identified by using Western blotting, and isolated by limiting dilution. Due to differences in promoter potency, the selected cell line that singly expresses ErbB-4 (E4 cells) contained approximately 10–12-fold more ErbB-4 molecules than cell lines expressing the combinations of ErbB-4 with ErbB-2 (D24 cells) or with ErbB-3 (D34 cells). A cell line expressing only approximately 5×10^4 ErbB-4 molecules per cell was established by using previously described procedures (29) and denoted D4.

Radiolabeling of Ligands, Covalent Cross-linking, and Ligand Binding Analyses—Growth factors were labeled by using IODO-GEN as described (32). The specific activity was approximately 5×10^5 cpm/ng. For covalent cross-linking analysis, cells (10^6) were incubated on ice for 1.5 h with ^{125}I-EGF, ^{125}I-NDF-β1, or ^{125}I-epiregulin (each at 100 ng/ml). The chemical cross-linking reagent BS^3 was then added (1 mM), and after 90 min on ice, cells were pelleted and solubilized in solubilization buffer. For analyses of ligand displacement with 32D cells, 10^6 cells were washed once with binding buffer, and then incubated for 2 h at 4 °C with a radiolabeled ligand (1 ng/ml) and various concentrations of an unlabeled ligand in a final volume of 0.2 ml. Nonspecific binding was determined in the presence of a 100-fold molar excess of the unlabeled ligand. To terminate ligand binding, each reaction tube was washed once with 0.5 ml of binding buffer and loaded on top of a 0.7-ml cushion of bovine serum. The tubes were spun (12,000 × *g*, 2 min) to remove the unbound ligand. Ligand displacement from CHO cells was analyzed with cell monolayers grown in 24-well dishes. Monolayers were washed once with binding buffer and then incubated for 2 h at 4 °C with 1 ng/ml of the radiolabeled ligand, along with increasing concentrations of an unlabeled growth factor. Then, cells were washed three times with ice-cold binding buffer. Labeled cells were lysed for 15 min at 37 °C in 0.5 ml of 0.1 N NaOH solution containing 0.1% sodium dodecyl sulfate, and the radioactivity was determined. Nonspecific binding was calculated by subtracting the binding of radiolabeled ligands to untransfected CHO cells, or by performing the binding assays in the presence of a 100-fold excess of the unlabeled ligand.

Receptor Down-regulation Assay—Ligand-induced receptor down-regulation was measured as follows: cells grown in 24-well plates were incubated at 37 °C for up to 90 min without or with various ligands in binding buffer. The cells were then put on ice, rinsed twice with binding buffer, and surface-bound ligand molecules removed by using a 7-min long incubation in 0.5 ml of solution of 150 mM acetic acid, pH 2.7, containing 150 mM NaCl (33). The number of ligand-binding sites that remained exposed on the cell surface was then determined by incubating cells at 4 °C with radiolabeled EGF (20 ng/ml) for 90 min.

Lysate Preparation and Western Blotting—For analysis of total cell lysates, gel sample buffer was added directly to cell monolayers or suspensions. For other experiments, solubilization buffer was added to cells on ice. The adherent CHO cells were scraped with a rubber policeman into 1 ml of buffer, transferred to microtubes, mixed harshly, and centrifuged (10,000 × *g*, 10 min at 4 °C). Samples were resolved by gel electrophoresis through 7.5% acrylamide gels, and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked for 2 h in TBST buffer (0.02 Tris-HCl buffered at pH 7.5, 0.15 M NaCl, and 0.05% Tween 20) containing 1% milk, blotted for 2 h with 1 μg/ml primary antibody, washed, and reblotted with 0.5 μg/ml secondary antibody linked to horseradish peroxidase. Immunoreactive bands were detected with an enhanced chemiluminescence reagent (Amersham Corp.).

Cell Proliferation Assays—Cells were washed free of IL-3, resuspended in RPMI 1640 medium at 5×10^5 cells/ml, and treated without or with growth factors or IL-3 (1:1000 dilution of medium conditioned by IL-3-producing cells). Cell survival was determined by using the MTT assay as described previously (29). MTT (0.1 mg/ml) was incubated for 2 h at 37 °C with the analyzed cells. Living cells can transform the tetrazolium ring into dark blue formazan crystals, that can be quantified by reading the optical density at 540–630 nm after lysis of the cells with acidic isopropyl alcohol (34).

Cellular Differentiation Assays—MDA-MB453 human mammary cancer cells were plated in chamber slides (Lab-Tek) and then incubated for 4 days in the absence or presence of ligands (50 ng/ml). Cells were stained with oil red O, to visualize neutral lipids, as described previously (35).

RESULTS

Induction of Cellular Differentiation and Tyrosine Phosphorylation by Epiregulin in the Absence of ErbB-1—The duality of epiregulin's activity, namely, mitogenicity for some normal

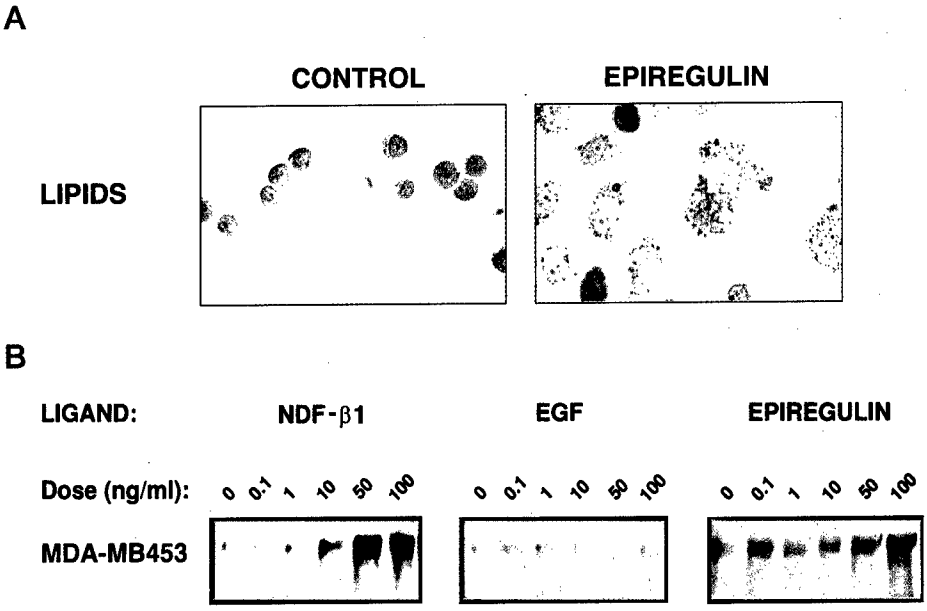

FIG. 1. **Induction of cellular differentiation and tyrosine phosphorylation by epiregulin in mammary cells lacking ErbB-1/EGF receptor.** *A*, MDA-MB453 human mammary cancer cells, that express no ErbB-1, were plated in chamber slides and then incubated for 4 days in the absence (*CONTROL*) or presence of epiregulin (50 ng/ml). Cells were stained with oil red O, to visualize neutral lipids. Note the appearance of lipid droplets (*yellow*) in epiregulin-treated cells. The magnification used was × 600. *B*, following an overnight starvation, 10^6 MDA-MB453 cells were incubated for 2 min at 37 °C without or with EGF, NDF-β1, or epiregulin, at the indicated concentrations. Whole cell lysates were then prepared, resolved by gel electrophoresis, and immunoblotted with an antibody to phosphotyrosine (PY20). Bound antibody was detected by using a chemiluminescence-based method.

cells and growth inhibition of epithelial tumor cells (21), may depend on expression patterns of ErbB proteins, and thus may be explained by epiregulin's interaction with receptor species other than ErbB-1. As an initial test of this paradigm we examined the biological effect of epiregulin on MDA-MB453 mammary tumor cells, which are devoid of the EGF-receptor (ErbB-1), but can undergo phenotypic differentiation in response to EGF-like ligands (36). Evidently, these cells underwent growth arrest in response to long-term incubation with epiregulin, and displayed phenotypic differentiation that included cell flattening, and appearance of neutral lipid-containing vesicles (Fig. 1*A*). EGF, at 1–200 ng/ml, was inactive in inducing cell differentiation (data not shown), whereas similar phenotypic alterations were induced also by NDF/neuregulin, a ligand that interacts with both ErbB-3 and ErbB-4 (37). Consistent with their biological effects on MDA-MB453 cells, both epiregulin and NDF, but not EGF, were able to stimulate tyrosine phosphorylation of a 180-kDa protein at concentrations higher than 10 ng/ml (Fig. 1*B*). In conclusion, epiregulin action on the mammary epithelial cell line we examined is independent of ErbB-1, and is distinct from the effect of EGF.

Epiregulin Is a Relatively Potent Stimulator of ErbB-1, but It Can Transmit Biological Signals Also through Combinations of Other Receptors—To directly address the specificity of epiregulin to ErbB receptors, we employed a previously described series of cell lines derived from the IL-3-dependent 32D myeloid cell line (29). Parental 32D cells express no ErbB protein, but as a result of transfection, the derivative lines singly express ErbB-1, ErbB-2, ErbB-3, or ErbB-4 (cell lines denoted D1, D2, D3, and E4, respectively). Likewise, co-expression of two ErbB proteins established cell lines with various combinations. For example, D13 cells co-express ErbB-1 and ErbB-3. Analysis of cell proliferation in the absence of IL-3, but in the presence of increasing concentrations of epiregulin, EGF, or NDF-β1, revealed several interesting characteristics of epiregulin. First, the factor was more potent than EGF on cells singly expressing ErbB-1 (D1 cells, Fig. 2*A*), as well as on cells expressing combinations of ErbB-1 with either ErbB-2 (D12 cells) or ErbB-3 (*D13 panel* in Fig. 2*A*). Not only were the dose-response curves of epiregulin shifted to the left, but this ligand exerted in D1 and D13 cells a higher maximal response than EGF. Consistent with the catalytic inactivity of ErbB-3 (38), and the inability of ErbB-2 to bind any of the ErbB ligands with high affinity (12), cells singly expressing ErbB-3 or ErbB-2 (D3 and D2 cell lines, respectively) did not respond to epiregulin (Fig. 2*A*). For control, we verified that D2 cells are stimulatable by a mAb to ErbB-2 (25) (Fig. 2*A*), and D3 cells retained response to IL-3 (Fig. 3). Surprisingly, E4 cells that highly overexpress ErbB-4 exhibited mitogenic response to both epiregulin and EGF at concentrations above 5 ng/ml (Fig. 2*A*). In fact, the response to EGF was reproducibly slightly higher than the mitogenic effect of epiregulin on these cells. Due to the use of different promoters, ErbB-4 expression in the E4 cell line was more than 10-fold higher than that of ErbB-1 in D1 cells (see "Experimental Procedures"). To address the possibility that epiregulin and EGF act through ErbB-4 only when this receptor is overexpressed, we analyzed a second cell line, D4, whose ErbB-4 expression is comparable with the level of ErbB-1 expression in D1 cells. When tested on D4 cells, both ligands displayed mitogenic activity (Fig. 2*B*), along with an ability to stimulate tyrosine phosphorylation (Fig. 2*C*). Nevertheless, in terms of the maximal response to IL-3, both epiregulin and EGF were more active on the ErbB-4-overexpressing cell line than on the low expressor D4 cells, implying that the level of expression of ErbB-4 affects the level of cell proliferation, but not ligand specificity.

Although the effect of epiregulin on cells coexpressing ErbB-3 with ErbB-1 (D13 cells) was higher than that of EGF, the response to NDF was much higher, presumably because NDF better recruits ErbB-3 into heterodimers (29, 39). Nevertheless, it is clear that also epiregulin can recruit ErbB-3 into heterodimers, as reflected by its activity on cells coexpressing a combination of ErbB-3 with either ErbB-2 (D23 cells, Fig. 2*A*) or ErbB-4 (D34 cells, Fig. 2*A*). This ability of epiregulin distinguishes it from EGF, whose signaling through the ErbB-2/ErbB-3 heterodimer occurs only at extremely high concentrations (Fig. 2*A* and Ref. 40 and 41), and is completely inactive in stimulating an ErbB-3/ErbB-4 heterodimer (Fig. 2*A*). Moreover, although EGF is slightly more potent than epiregulin on ErbB-4-expressing cells (*E4 panels* in Figs. 2*A* and 3), epiregulin is superior when ErbB-2 is coexpressed with ErbB-4 (*D24 panels* in Figs. 2*A* and 3), suggesting that this ligand is a better stimulator of the ErbB-2/ErbB-4 heterodimer. Taken together, the results presented in Fig. 2 imply that relative to EGF, epiregulin is a better agonist of ErbB-1-containing homo- and heterodimers. In addition, recruitment of ErbB-2, ErbB-3, and ErbB-4 into heterodimers is more efficient in the case of epiregulin. However, homodimers of ErbB-4 are better activated

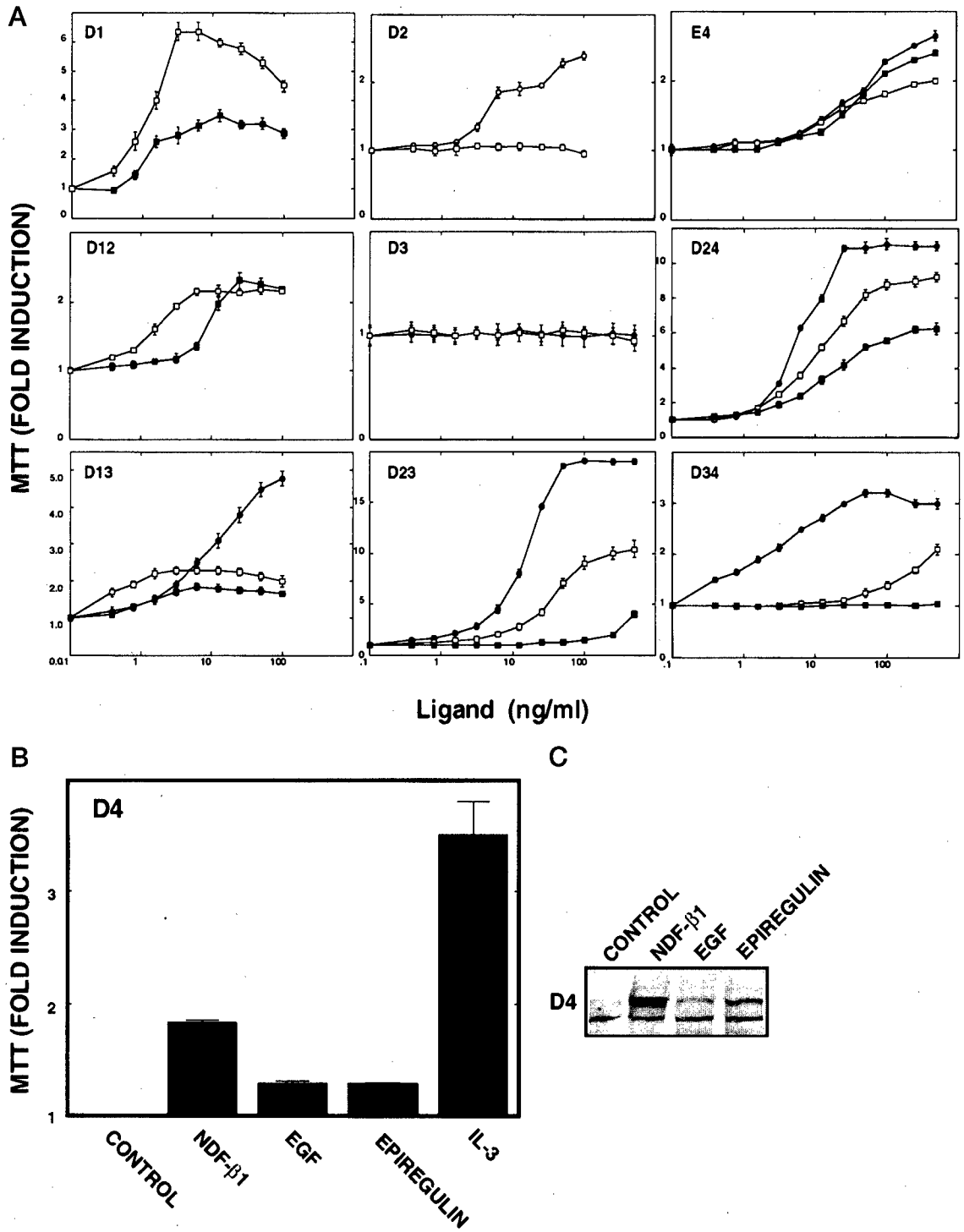

FIG. 2. **Proliferative responses of ErbB-expressing derivatives of 32D myeloid cells to epiregulin and other ligands.** *A,* the indicated sublines of 32D cells were tested for cell proliferation by using the MTT assay. D1, D2, D3, and E4 cells express ErbB-1, ErbB-2, ErbB-3, and ErbB-4, respectively, whereas the other cell lines co-express the corresponding two ErbB proteins. Cells were deprived of IL-3 and plated at a density of 5×10^5 cells/ml in media containing serial dilutions of EGF (*closed squares*), epiregulin (*open squares*), NDF-β1 (*closed circles*), or a monoclonal antibody to ErbB-2 (mAb L26, *open circles*). The MTT assay was performed 24 h later. Results are presented as fold induction over the control untreated cells, and are the mean ± S.D. of four determinations. Each experiment was repeated at least twice. Cells singly expressing ErbB-3 (D3 cells) responded to none of the ligands we tested, but these cells retained response to IL-3. *B,* D4 cells were tested for cell proliferation by using the MTT assay as described above, except that the indicated ligands were used at 100 ng/ml. For control, cells were incubated in the absence of IL-3 or ligands. *C,* ligand-induced tyrosine phosphorylation was analyzed in D4 cells by incubating 10^6 cells without or with the indicated ligands (each at 100 ng/ml). Following 2 min at 37 °C, whole cell lysates were prepared and analyzed by immunoblotting with a mAb to phosphotyrosine. Antibody detection was performed with a chemiluminescence kit. Only the 180-kDa region of the blot is shown.

by EGF, and neither homodimers of ErbB-2 nor ErbB-3·ErbB-3 complexes are stimulatable by the two ligands.

These conclusions were further supported by long-term survival experiments that are presented in Fig. 3. In this type of analysis cells are maintained in the absence of IL-3, but in the presence of epiregulin (or other ligands) for several days, and

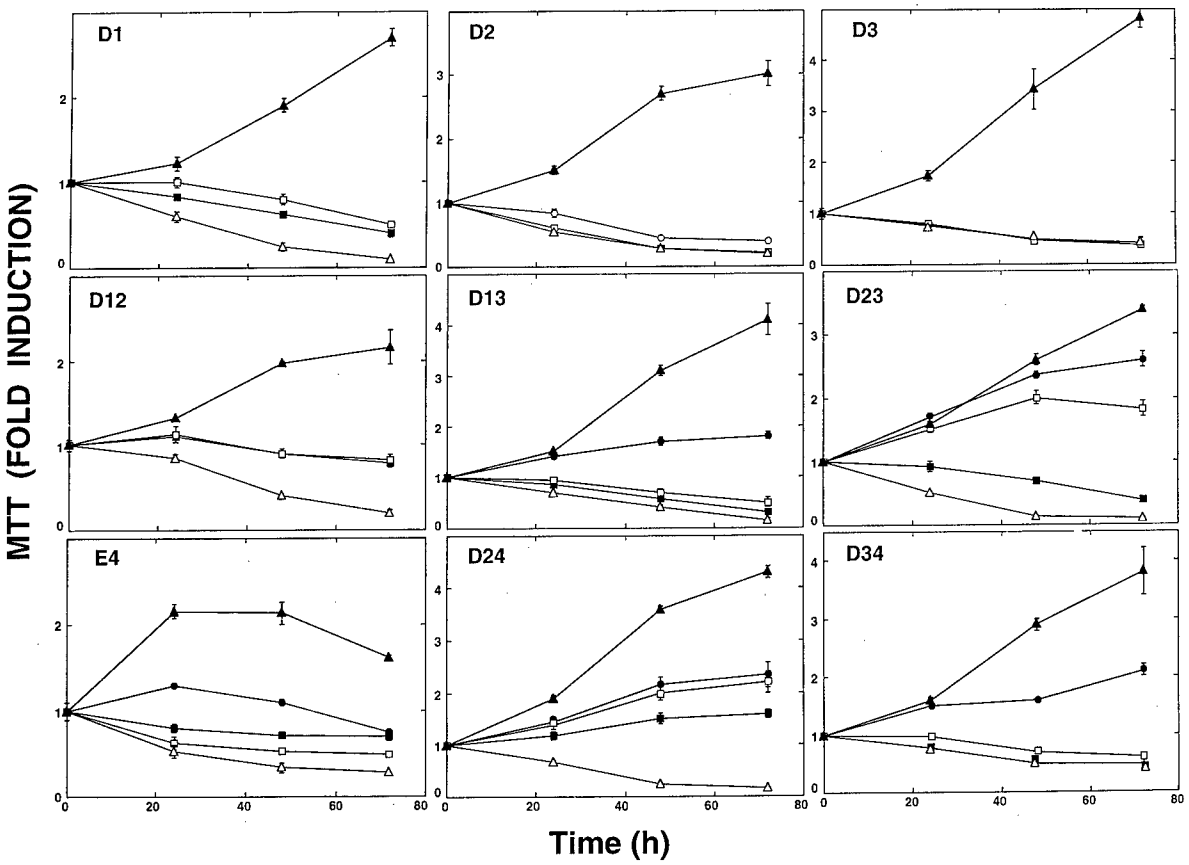

FIG. 3. **Ligand-dependent survival of ErbB-expressing 32D cells in the absence of IL-3.** The indicated sublines of 32D cells were incubated for various time intervals at a density of 5×10^5 cells/ml in the presence (*closed triangles*) or absence of IL-3 (*open triangles*), or with one of the following ligands, each at a concentration of 100 ng/ml (except for D23 cells, that were treated with EGF at 500 ng/ml to reflect the residual activity of this ligand through the ErbB-2/ErbB-3 heterodimer (40, 41)): EGF (*closed squares*), epiregulin (*open squares*), NDF-β1 (*closed circles*), and an antibody to ErbB-2 (mAb L26, *open circles*). Cell survival was determined daily by using the colorimetric MTT assay. The data presented are the mean ± S.D. of six determinations. Each experiment was repeated at least twice.

their survival determined by using the MTT assay. Consistent with the dose curves of the short-term mitogenic assay, at a saturating concentration epiregulin acted as a slightly better survival factor than EGF for cells expressing ErbB-1, either alone or in combination with ErbB-3 (Fig. 3). Also consistent with the data of Fig. 2 was the observation that EGF exerted a better survival activity on ErbB-4-overexpressing cells (*E4 panel* in Fig. 3). Interestingly, the presence of ErbB-2, together with either ErbB-4 or ErbB-3, enabled epiregulin to become a potent stimulator of cell proliferation, whereas EGF acted primarily as a survival factor under these circumstances (*D23* and *D24 panels* in Fig. 3). Although survival of cells coexpressing ErbB-3 and ErbB-4 was only slightly extended by epiregulin (*D34 panel* in Fig. 3), this effect was higher than that of EGF, reinforcing the relative preference of epiregulin for heterodimeric receptor combinations.

Receptor Phosphorylation and MAP Kinase Activation by Epiregulin—Signaling by all EGF-like ligands is mediated by rapid tyrosine phosphorylation of the respective receptors, and is ultimately funneled to the mitogen-activated protein kinase (MAP-kinase/Erk) pathway (42). The biological differences we observed between epiregulin, EGF, and NDF in subsets of 32D cells suggested that these ligands may differ in signaling potency, and especially in their ability to recruit the MAPK pathway. To analyze receptor phosphorylation and MAPK activation we probed blots of whole extracts, prepared from ligand-stimulated cells, with antibodies to phosphotyrosine, or with a murine mAb that specifically recognizes the active, doubly phosphorylated form of the ERK1 and ERK2 MAPKs (26). Surprisingly, the more mitogenic ligand of ErbB-1, epiregulin, exhibited weaker, but not less sustained, tyrosine phosphorylation of proteins at the 180-kDa range corresponding to ErbB-1 in D1 cells (Fig. 4*A*). Although both EGF and epiregulin stimulated MAPK phosphorylation in these cells, the patterns of activation differed: a comparable increase in the activity of both forms of the kinase was induced by epiregulin, whereas primarily the lower form was activated after stimulation with EGF. Importantly, although stimulation by EGF was more uniform at intermediate time intervals (10–20 min), it completely disappeared after 30–60 min, at which time the effect of epiregulin was still detectable. By contrast, ErbB-4 phosphorylation was stronger with epiregulin than with EGF (*E4 panel* in Fig. 4*A*), although the latter is a slightly more efficient mitogen for the ErbB-4-overexpressing E4 cells (Figs. 2*A* and 3). These differences between ErbB-1 and ErbB-4 phosphorylation are cell-type independent, because they were reproduced in a series of CHO cells expressing ErbB-1 (CB1 cells) or ErbB-4 (CB4 cells), on a low background of the endogenous hamster ErbB-2 (Fig. 4*B*). Analysis of 32D cells expressing a combination of ErbB-2 with ErbB-3 (D23 cells) revealed that both forms of MAPK were rapidly stimulated by epiregulin, but phosphorylation of both ErbBs and MAPKs by EGF occurred only at very high ligand concentrations, in agreement with recent reports (40, 41). The maximal activation of MAPK in these cells was observed upon stimulation with NDF, a ligand whose mitogenic effect was almost equivalent to that of IL-3 (Fig. 3). A relatively sustained stimulation, and appearance of an activated Erk-2, were observed upon activation of both D23 and D24 cells by their most potent ligand, namely, NDF, implying that these features may characterize the stronger mito-

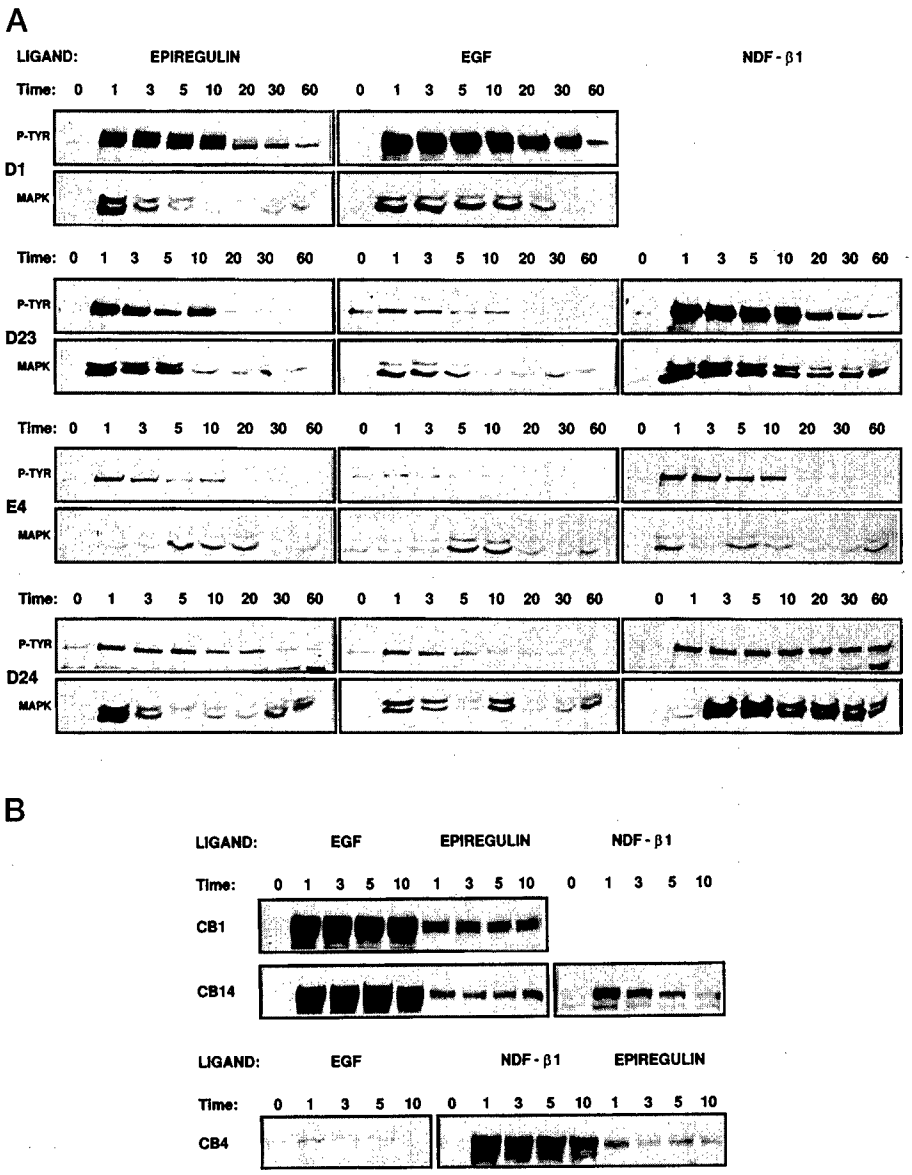

FIG. 4. **Kinetics of receptor phosphorylation and MAP kinase activation by epiregulin and other ligands.** The following derivatives of 32D cells (D or E series of cell lines, *panel A*), or CHO cells (CB series of cell lines, *panel B*), were incubated at 37 °C for various time intervals (indicated in minutes) with epiregulin (100 ng/ml), EGF (100 ng/ml, except for D23 cells that were treated with 500 ng/ml), or NDF-β1 (100 ng/ml): D1, CB1, E4, and CB4 cells that singly express ErbB-1 or ErbB-4, respectively, whereas D23, D24, and CB14 cells co-express a combination of the corresponding two receptors. In the end of the incubation period, whole cell lysates were prepared, cleared from debris and nuclei, resolved by gel electrophoresis, and subjected to immunoblotting with either an antibody to phosphotyrosine (*P-TYR*), or with an antibody specific to the active doubly phosphorylated form of MAPK, as indicated. Derivatives of CHO cells were analyzed only with antibodies to phosphotyrosine. Signal detection was performed by using a chemiluminescence kit.

genic signals. In conclusion, the relative strength of mitogenic signals of EGF-like ligands better correlates with the duration of MAPK activation (especially the modification of Erk-2) than with the intensity of ErbB phosphorylation.

Low Affinity Interaction of Epiregulin with ErbB-1 and Other ErbB Proteins—The relatively weak stimulation of ErbB-1 phosphorylation by epiregulin (*D1 panel* in Fig. 4) suggested low affinity interaction of epiregulin with ErbB-1 on D1 cells. This possibility was addressed by employing two assays: covalent cross-linking of a radiolabeled epiregulin to the surface of ErbB-expressing 32D cell derivatives (Fig. 5), and ligand displacement analyses that were performed with both 32D- and CHO-derived cell lines (Fig. 6). Epiregulin was radiolabeled with ^{125}I and covalently cross-linked to the surface of 32D cells by using the BS^3 covalent cross-linking reagent. The specificity of labeling by epiregulin was evident by the absence of covalent cross-linking to ErbB-2 and ErbB-3, when these receptors were singly expressed (*D2* and *D3 cells*, respectively, Fig. 5), and by displacement of radioactive epiregulin by a large excess of the unlabeled ligand (data not shown). Interestingly, only a very weak signal was observed when radiolabeled epiregulin was covalently cross-linked to cells singly expressing ErbB-1, although these cells displayed a strong cross-linking signal with ^{125}I-EGF, whose specific radioactivity was comparable to that of ^{125}I-epiregulin (Fig. 5). A slightly stronger signal was observed when cells coexpressing ErbB-1 and ErbB-2 were analyzed, implying that the corresponding heterodimer cooperatively interacts with epiregulin. The combination of ErbB-1 with ErbB-3 was less efficient than that of ErbB-1 with ErbB-2, although the numbers of ErbB-1 molecules on D1, D12, and D13 cells were similar. By contrast with ErbB-1, affinity labeling of ErbB-4 in the overexpressing E4 cell line was very efficient in the case of both epiregulin and NDF, but relatively weak labeling was observed with EGF (Fig. 5), in accordance with receptor phosphorylation signals (Fig. 4*A*). Similar observations were made with the D4 and CB4 cell lines (data not shown). Interestingly, we were unable to detect covalent cross-linking of epiregulin to cells coexpressing ErbB-3 with either ErbB-2 or ErbB-4 (*D23* and *D34 lanes* in Fig. 5, note that ErbB-4 expression in D24 and D34 cells is approximately 10-fold lower than in E4 cells), although these combinations reacted with NDF. By contrast, the ErbB-2/ErbB-4 combination displayed a clearly detectable signal with ^{125}I-epiregulin, reflecting the relatively high mitogenic response of D24 cells to epiregulin (Figs. 2*A* and 3).

We then compared the capacity of epiregulin, as opposed to EGF, to displace a cell-bound radioactive EGF from the surface of 32D or CHO cells singly expressing ErbB-1 (D1 and CB1

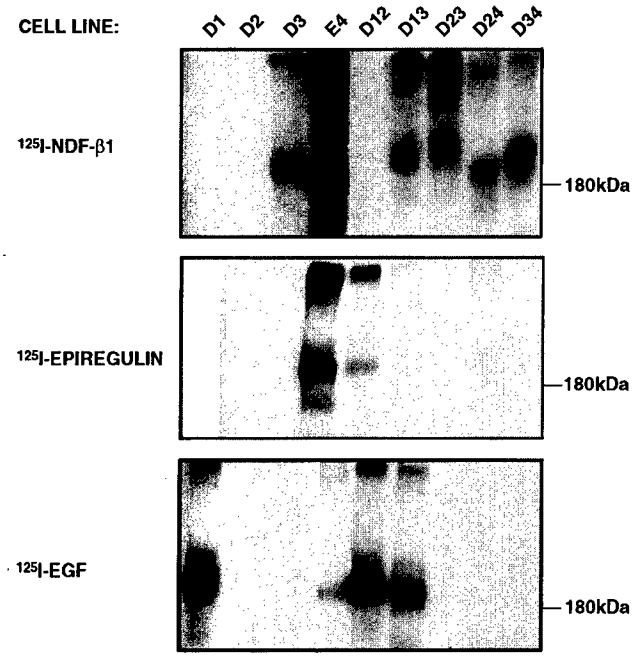

FIG. 5. **Covalent cross-linking of radiolabeled epiregulin and other ligands to ErbB-expressing cells.** The indicated derivatives of 32D myeloid cells (10^6 cells per lane) were incubated at 4 °C with ^{125}I-EGF, ^{125}I-epiregulin, or ^{125}I-NDF-β1, each at 100 ng/ml. Following 90 min of incubation, the covalent cross-linking reagent BS^3 was added (1 mM final concentration), and cell lysates prepared after an additional 1.5 h of incubation. Lysates were resolved by gel electrophoresis and autoradiography. The location of a 180-kDa molecular weight marker is indicated.

cells, respectively). In contrast with the mitogenic superiority of epiregulin for ErbB-1-expressing 32D cells, the apparent binding affinity of epiregulin, as reflected by the competition curves, was 2 orders of magnitude lower than that of EGF (*D1* and *CB1 panels* in Fig. 6, *A* and *B*). The presence of ErbB-4 together with ErbB-1 did not significantly alter the ability of epiregulin to displace EGF from the surface of CB14 cells (Fig. 6*B*), although epiregulin was able to displace, albeit with low efficiency, a surface-bound ^{125}I-NDF from ErbB-4-expressing cells (*D4* or *CB4 cells*, Fig. 6, *A* and *B*). The results of ligand displacement experiments that were performed with E4 cells were qualitatively similar (data not shown). NDF displacement by epiregulin, or EGF, was relatively efficient in D24 cells, but only weak competition was detectable in D34 cells, consistent with the relative mitogenic potency of epiregulin for D24 and D34 cells (Figs. 2*A* and 3). Thus, affinity labeling (Fig. 5) and ligand competition analyses (Fig. 6) imply that epiregulin binds cooperatively to the combination of ErbB-2 with ErbB-4. By contrast, only very weak competition between epiregulin and NDF was observed in cells expressing ErbB-3, either alone or in combination with ErbB-2 or ErbB-4 (Fig. 6*A*), implying that ErbB-3, unlike ErbB-4, does not cooperate with ErbB-2 in epiregulin binding. This conclusion is consistent with the absence of a detectable cross-linking signal in D3, D13, and D23 cells (Fig. 5). In light of this inference the results obtained with D23 cells are interesting because epiregulin displayed only a slightly better ability than EGF to displace NDF from these cells, but its mitogenic activity was much stronger than that of EGF (Figs. 2*A* and 3). In conclusion, receptor binding analyses indicated direct interaction between epiregulin and two receptors, ErbB-1 and ErbB-4. Although neither ErbB-3 nor ErbB-2 directly interact with epiregulin, the latter protein significantly cooperates with both direct receptors of epiregulin.

Epiregulin-induced Down-regulation of ErbB-1 Is Defective—The superior mitogenic activity of epiregulin is analogous to that of TGFα. This latter ligand of ErbB-1 is a better agonist than EGF when tested *in vitro* in mitogenic, angiogenic, and motogenic assays (43, 44). Apparently, the relatively potent activity of TGFα, whose binding affinity is almost identical to that of EGF, is due to the absence of receptor down-regulation, which allows sustained cellular activation (45). To examine the possibility that epiregulin's superiority is due to a defective receptor inactivation process, we exposed CB1 cells to epiregulin, EGF, or TGFα, and determined the extent of disappearance of ErbB-1 from the cell surface. Evidently, whereas EGF induced gradual disappearance of the surface-exposed ErbB-1, neither epiregulin nor TGFα led to a significant change in the level of surface ErbB-1 (Fig. 7), although at the concentrations we used both ligands were more mitogenic than EGF (Fig. 2*A*, and data not shown). In experiments that are not presented we found that the difference in receptor down-regulation was not due to defective endocytosis of epiregulin, whose rate of internalization was comparable to that of EGF and TGFα. This observation raised the possibility that unlike EGF, which directs ErbB-1 to degradation in lysosomes, epiregulin binding to ErbB-1 is followed by receptor recycling, a route taken by the TGFα-driven ErbB-1 (45, 46). This notion was supported by an experiment that tested the effect of monensin, a well characterized inhibitor of receptor recycling (47), on down-regulation of ErbB-1. In the presence of the carboxylic ionophore both epiregulin and TGFα induced significant down-regulation of ErbB-1, but this compound was ineffective on the extensive down-regulation that was induced by EGF (Fig. 7, and data not shown). In conclusion, the relatively strong biological action of epiregulin through ErbB-1 may be due to continuous recycling of ErbB-1 back to the cell surface, thus allowing prolongation of epiregulin signaling.

DISCUSSION

The evolutionary pathway of the ErbB signaling module, from worms (48) and flies (49) to mammals, indicates that duplication of genes encoding EGF-like ligands preceded multiplication of receptor-encoding genes. Despite multiplicity of ligands and receptors, it is clear that the downstream signaling mechanisms, namely a linear cascade leading to MAPK activation, has been conserved. Thus, to gain functional diversity, variations on the common theme of ligand-ErbB-MAPK evolved throughout evolution. Examination of the interactions between one of the mammalian ErbB ligands, epiregulin, and various combinations of the four ErbB proteins uncovered two novel features of the evolved module, that are schematically presented in Fig. 8. First, epiregulin is a broad-specificity ligand that activates all eight ligand-stimulatable combinations of ErbBs. Second, despite its extremely low affinity, signaling by epiregulin is more potent than the bioactivity of a high affinity ligand, namely, EGF. The mechanisms underlying these two features, and their functional implications, are discussed below.

Pan-ErbB Specificity of Epiregulin—The four mammalian ErbB proteins can form 10 homo- and heterodimeric complexes, including an ErbB-3 homodimer, which is biologically inactive (29), and an ErbB-2 homodimer whose formation may be driven by receptor overexpression (50), or by a transmembrane oncogenic mutation (51). Epiregulin can signal through all but these two homodimeric combinations of ErbBs (Fig. 8). This broad specificity is unique; no other EGF-like ligand has such a wide selection of receptors. However, due to its broad selectivity, none of the receptors of epiregulin binds it with high affinity (Figs. 5 and 6).

One of the most surprising observations made in the course of the present study is the ability of both epiregulin and EGF to activate ErbB-4 when this receptor is singly expressed. This

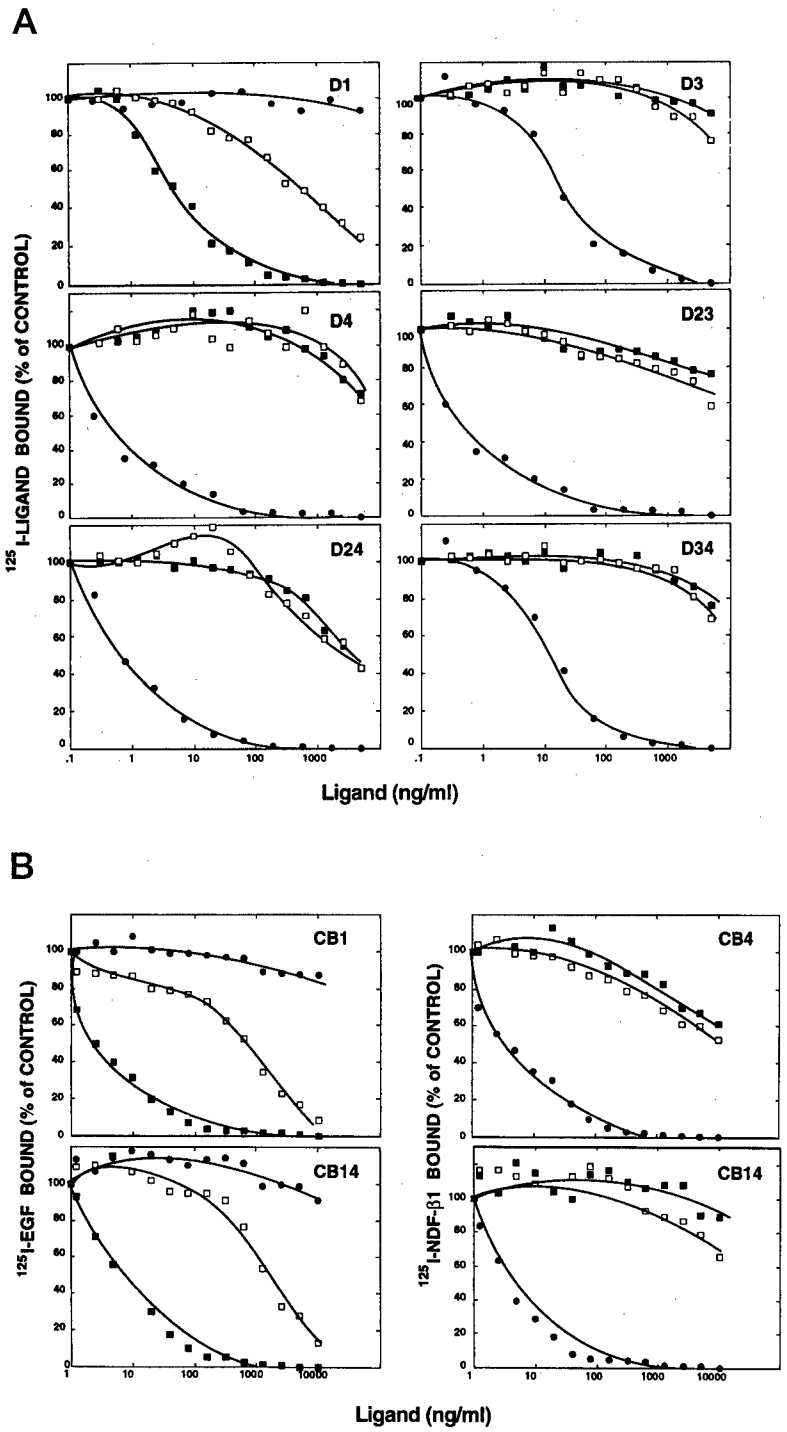

FIG. 6. **Binding of epiregulin to cell lines expressing specific ErbB proteins and their combinations.** Ligand displacement analyses were performed with derivatives of 32D myeloid cells (D series of cell lines, *panel A*), or with CHO cells expressing ErbB-1, ErbB-4, or their combinations (CB series of cell lines, *panel B*). Either radiolabeled EGF (*D1, CB1,* and the *left-hand CB14 panel*) or radioactive NDF-β1 (*D3, D4, D23, D24, D34, CB4,* and the *right-hand CB14 panel*) were used. Cells (10^6) were incubated for 2 h at 4 °C with the radiolabeled ligand (1 ng/ml) in the presence of increasing concentrations of an unlabeled epiregulin (*open squares*), EGF (*closed squares*), or NDF-β1 (*closed circles*). Each data point represents the mean (less than 10% variation) of two determinations.

observation is reminiscent of several recent reports that identified betacellulin (9) and heparin-binding EGF (10, 11) as ligands of ErbB-4. Conceivably, ErbB-1 and ErbB-4 share some structural features at their ligand-binding sites, thus defining a subgroup of direct ErbB-1 ligands, including EGF, betacellulin, and heparin-binding EGF, but excluding TGFα and amphiregulin, as ligands with dual receptor specificity. Nevertheless, like all other interactions of epiregulin, binding to ErbB-4 is characterized by very low affinity; the corresponding dissociation constant is estimated to be in the micromolar range (*D4* and *CB4 panels* in Fig. 6). The affinity of the other direct receptor of epiregulin, ErbB-1, is only 10-fold better, much higher than the nanomolar or lower apparent K_d of EGF or NDF binding to their direct receptors (Fig. 6*A*). However, receptor combinations containing ErbB-1 and ErbB-4 are not the only receptors for epiregulin; although this ligand does not interact with isolated components of the ErbB-2/ErbB-3 heterodimer, it can efficiently stimulate the respective receptor combination (*D23 panels* in Figs. 2*A* and 3). This is probably mediated by an extremely low affinity of epiregulin to ErbB-3 (*D3 panel* in Fig. 6), and by a cooperative effect of the coexpressed ErbB-2. This effect of the ligand-less ErbB-2 is extended to heterodimers containing the direct epiregulin receptors, namely ErbB-1 and ErbB-4; cooperativity is exemplified by the relatively strong binding of epiregulin to cells coexpressing ErbB-1 and ErbB-2 (but not to cells co-expressing ErbB-1 and ErbB-3, Fig. 5), and by the ability of ErbB-2 to augment epiregulin binding to ErbB-4 (compare *D4* and *D24 panels* in

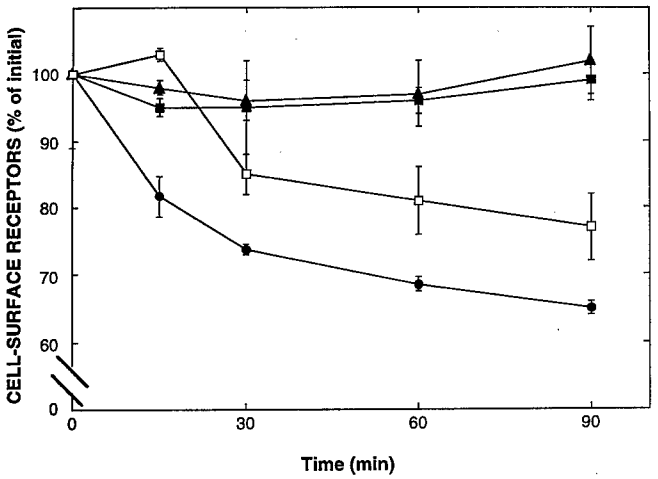

FIG. 7. **Epiregulin-induced down-regulation of ErbB-1.** CB1 cells were grown to 80% confluence in 24-well plates, rinsed with binding buffer, and incubated at 37 °C for the indicated time intervals with one of the following ligands (each at 1 ng/ml): epiregulin (*closed squares*), EGF (*circles*), or TGF-α (*triangles*). Sister epiregulin-treated cells were similarly incubated, except that monensin (0.1 mM) was added to the medium (*open squares*). Thereafter, monolayers were rinsed twice with binding buffer, followed by a 7-min long incubation with a low pH stripping buffer that removes surface-bound ligands. The level of surface receptors, relative to the number of ligand-binding sites present before down-regulation, was determined by incubating cells for 1.5 h at 4 °C with radiolabeled EGF. The results are expressed as the average fraction and range (*bars*) of the original binding sites that remained on the cell surface after exposure to the non-labeled ligand at 37 °C.

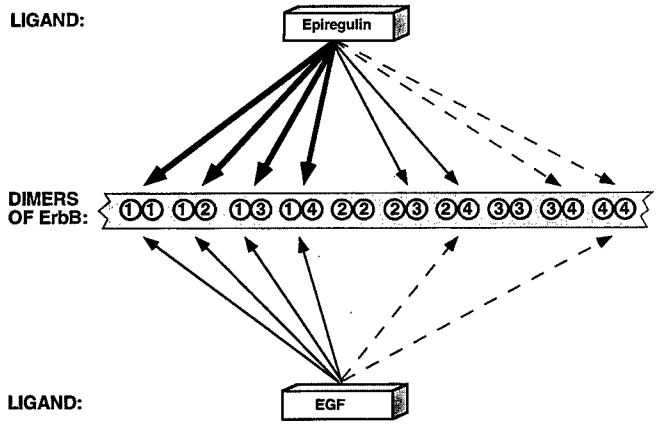

FIG. 8. **Summary of epiregulin-receptor interactions.** The *horizontal gray bar* represents the plasma membrane, and the 10 possible receptor dimers are shown schematically as double circular structures. Specific ErbB proteins are identified by their *numbers*. Two ErbB ligands, epiregulin and EGF, are compared and their relative strength of signaling, as revealed by using an IL-3-dependent series of cell lines, are represented by the thickness of the corresponding *arrows*. *Broken arrows* indicate very low bioactivity. For simplicity, the ability of EGF to stimulate an ErbB-2/ErbB-3 heterodimer at very high ligand concentrations is not represented. Note that no ligand binds with high affinity to ErbB-2 homodimers. Because no 32D cell derivative co-expressing ErbB-1 and ErbB-4 has been established, the data related to this heterodimeric combination was inferred from experiments with transfected CHO cells. All other receptor combinations were examined in 32D cell derivatives.

Fig. 6). This binding effect is translated to enhanced signaling by the ErbB-2/ErbB-4 heterodimer relative to the ErbB-4 homodimer, and is apparently more relevant to epiregulin than to EGF (compare *E4* and *D24 panels* in Fig. 2*A*). The mechanism underlying signal amplification by ErbB-2, a process that is significant to tumors overexpressing this receptor, has been previously attributed to its ability to decelerate dissociation of NDF and EGF from ErbB-2-containing heterodimers (25, 52). The present study apparently extends this mechanism to epiregulin.

How does epiregulin recognize all six heterodimeric complexes of ErbBs? According to a ligand bivalence model (12), a notion supported by recent affinity labeling studies (53), and by measurements of the stoichiometry of ligand-receptor interactions in solution (54), epiregulin carries a high affinity binding site whose specificity is limited to ErbB-1 and ErbB-4. Another site that is structurally distinct and may localize to the C-terminal half of the ligand, binds with broad specificity but low affinity to other ErbB proteins, including ErbB-1 and ErbB-4 (thus allowing homodimer formation), as well as to ErbB-2 and ErbB-3, to confer heterodimer formation. Nevertheless, as is the case with EGF and NDF, the putative "low-affinity/broad-specificity" site of epiregulin apparently prefers ErbB-2 over other receptors. This model explains how ErbB-2 augments epiregulin signaling through the ErbB-2/ErbB-3 and ErbB-2/ErbB-4 heterodimers.

Mechanism of Signaling Superiority of Low Affinity Ligand-ErbB Interactions—In their original analysis of epiregulin interactions with various cell types, Toyoda and collaborators (21) found that this ligand was more mitogenic than EGF for several types of normal cells, although epiregulin binding to cells of another type (the A-431 epidermoid carcinoma line) displayed a 10-fold lower affinity. Potentially, this discrepancy could be due to the different repertoires of ErbB proteins expressed on the surface of the different lines of cultured cells that these authors examined. However, our studies with engineered myeloid cells excluded this possibility, because epiregulin's superiority was retained also by cells singly expressing ErbB-1. In fact, our results extend the discrepancy between binding affinity and bioactivity to signaling through ErbB-4. Thus, epiregulin is a relatively potent stimulator of mitogenesis through both ErbB-1 and ErbB-4, despite being a very low affinity ligand of these two receptors (*D1* and *E4 panels* in Figs. 2*A* and 6*A*). The observation that ErbB-1 phosphorylation by epiregulin is weaker than the effect of EGF (Fig. 4*A*), implies that receptor activation is not the sole determinant of signaling potency. Instead, differences in the inactivation process may be critical: apart from differential recruitment of both tyrosine-specific phosphatases (55) and the negative regulator c-Cbl (56), endocytosis of ligand-receptor complexes is a major process that leads to inactivation of growth factor signaling (reviewed in Ref. 57). Our initial studies of this aspect of epiregulin's action indicated that this ligand, unlike EGF, mediates limited, if any, down-regulation of ErbB-1 (Fig. 7). Additional analyses raised the possibility that epiregulin undergoes internalization, but its receptor rapidly recycles to the cell surface (Fig. 7). Presumably, the very low affinity of epiregulin to ErbB-1 is insufficient to direct this receptor to lysosomal degradation, either because phosphorylation on tyrosine residues, which is essential for rapid internalization (58), is relatively inefficient, or because the ligand dissociates very rapidly. It is relevant that mutations of another receptor, that stabilize ligand-receptor interactions at the moderately acidic conditions of early endosomes, accelerate receptor degradation and prevent recycling (59, 60), indicating that the strength of ligand binding is critical for receptor routing. This mechanism of epiregulin/ErbB-1 interactions is expected to promote a relatively weak level of receptor activation, but due to receptor recycling, repeated association-dissociation cycles may result in prolongation of signaling. In support of this model, we observed an overall lower activation of MAPK by epiregulin, but this was more prolonged than in the case of EGF (*D1 panel* in Fig. 4*A*). Variations of the proposed mechanism have previ-

ously been reported: in the case of TGFα, whose binding affinity is comparable to that of EGF, the more rapid dissociation of the ligand-receptor complex in an acidic endosomal compartment drives ErbB-1 to recycling (45). This is contrasted with the lysosomal destination taken by an EGF-bound ErbB-1. As a result, signaling by TGFα is often more potent than that of EGF. An even closer example is provided by a mutant of EGF that was engineered to enhance the mitogenic potency of the growth factor for biotechnological applications (61). This mutant achieved mitogenic superiority through a combination of a 50-fold lower affinity, longer retention in culture supernatants, and a very limited receptor down-regulation.

In addition to the question how wide is the relevance of our findings to other growth factors whose binding affinities are very low, several other interesting questions are left open. The exceptionally broad specificity of epiregulin joins other observations that collectively imply non-redundancy of the multiple EGF-like ligands (reviewed in Ref. 62). Evidently, each ligand differs from other members of its family by a unique preference for certain ErbB proteins. This, however, does not explain how different ligands mediate mitogenesis on some cells, but differentiation (37), survival (63), or cell motility (10) on other types of cells, although in all cases the MAPK pathway is recruited. Even more difficult to reason is the inhibitory activity of epiregulin on certain epithelial cell lines (21), because all of its receptors turned out to be stimulatory for myeloid cells (Figs. 2*A* and 3). Perhaps a cell type-specific component lying downstream of ErbBs determines the nature of cellular response. Another puzzling issue is the contrast between the broad selectivity of epiregulin for ErbBs, and its very limited pattern of expression (23). This and other questions will require *in vivo* studies of epiregulin's physiological role.

Acknowledgments—We thank Barry Ratzkin (Amgen, Thousand Oaks, CA) for the recombinant NDF preparation, Rony Seger for monoclonal antibodies to activated MAP kinase, and Leah Klapper for the L26 antibody to ErbB-2.

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The EMBO Journal Vol.17 No.12 pp.3385–3397, 1998

Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers

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Both homo- and hetero-dimers of ErbB receptor tyrosine kinases mediate signaling by a large group of epidermal growth factor (EGF)-like ligands. However, some ligands are more potent than others, although they bind to the same direct receptor. In addition, signaling by receptor heterodimers is superior to homodimers. We addressed the mechanism underlying these two features of signal tuning by using three ligands: EGF; transforming growth factor α (TGFα); and their chimera, denoted E4T, which act on cells singly expressing ErbB-1 as a weak, a strong, and a very strong agonist, respectively. Co-expression of ErbB-2, a developmentally important co-receptor whose expression is frequently elevated in human cancers, specifically potentiated EGF signaling to the level achieved by TGFα, an effect that was partially mimicked by ErbB-3. Analysis of the mechanism underlying this *trans*-potentiation implied that EGF-driven homodimers of ErbB-1 are destined for intracellular degradation, whereas the corresponding heterodimers with ErbB-2 or with ErbB-3, dissociate in the early endosome. As a consequence, in the presence of either co-receptor, ErbB-1 is recycled to the cell surface and its signaling is enhanced. This latter route is followed by TGFα-driven homodimers of ErbB-1, and also by E4T-bound receptors, whose signaling is further enhanced by repeated cycles of binding and dissociation from the receptors. We conclude that alternative endocytic routes of homo- and hetero-dimeric receptor complexes may contribute to tuning and diversification of signal transduction. In addition, the ability of ErbB-2 to shunt ligand-activated receptors to recycling may explain, in part, its oncogenic potential.

Keywords: endocytosis/ErbB/HER family/oncogene/signal transduction/transforming growth factor α

Introduction

A large group of polypeptide growth factors mediates intercellular signaling by binding to, and activation of, transmembrane allosteric kinases with specificity to tyrosine residues (van der Geer *et al.*, 1994). As in other allosteric systems, the monomeric form of the receptor tyrosine kinase (RTK) is inactive, but upon ligand-induced oligomerization (primarily dimerization) it initiates a plethora of intracellular events ranging from stimulation of ion fluxes to cytoskeletal alterations, and culminating in regulation of gene expression. The underlying biochemical mechanism involves autophosphorylation of specific tyrosine residues of the activated receptor. These are turned into docking sites for cytoplasmic signaling proteins containing Src-homology 2 (SH-2) domains (Koch *et al.*, 1991), such as the adapter molecules SHC, Sem-5/Grb-2 and the p85 subunit of phosphatidylinositol 3′ kinase (Eagan and Weinberg, 1993). As a consequence thereof, several linear cascades of protein kinases are triggered, including the mitogen-activated protein kinase (MAPK) pathway (Seger and Krebs, 1995) and the S6-kinase pathway (Ming *et al.*, 1994).

In addition to this 'vertical' transduction pathway, lateral propagation of growth factor signals is made possible within subgroups of homologous RTKs by means of receptor heterodimerization. The best characterized example of 'lateral' signaling is provided by the type I RTKs (also named ErbB or HER family) (Carraway and Cantley, 1994; Alroy and Yarden, 1997). This subfamily comprises four members whose prototype is ErbB-1, a receptor that binds several ligands, including epidermal growth factor (EGF) and transforming growth factor (TGFα). Likewise, ErbB-3 and ErbB-4 bind three groups of alternatively spliced growth factors, collectively called neuregulins (Burden and Yarden, 1997). The fourth member, ErbB-2, binds no known ligand with high affinity. Nevertheless, impairment of ErbB-2 function by gene targeting resulted in a phenotype shared with that of neuregulin- and ErbB-4-deficient embryos (Lee *et al.*, 1995), and a mutant form of this receptor promotes cancer in rodents (Bargmann *et al.*, 1986). Overexpression of the wild-type human protein leads to phenotypic transformation of cultured cells (Di Fiore *et al.*, 1987; Hudziak *et al.*, 1987), and is frequently observed in several types of human carcinomas (Slamon *et al.*, 1987, 1989). Moreover, ErbB-2 overexpression predicts poor prognosis and resistance to certain therapeutic modalities, implying that the orphan receptor contributes to tumor virulence (reviewed in Hynes and Stern, 1994; Stancovski *et al.*, 1994). Despite the absence of a direct ligand, ErbB-2 plays a central role in a network of inter-receptor interactions; although the four ErbBs can form all 10 possible homo- and hetero-dimeric combinations, ErbB-2-containing heterodimers are preferred over other combinations (Tzahar *et al.*, 1996; Graus-Porta *et al.*, 1997). Each dimeric receptor complex has a distinct signaling potency, resulting in diversification and fine-tuning of signaling (Riese *et al.*, 1995; Pinkas-

Kramarski *et al.*, 1996a). In general, signaling by homodimeric complexes is relatively weak, whereas heterodimers, and especially those containing ErbB-2, are more potent transmitters of signals. The collaborative action of two different ErbBs is best exemplified by the potent combination of ErbB-2, the ligandless receptor, with ErbB-3, whose kinase function is defective, and is reflected by the synergistic effect on cell transformation of certain co-expressed pairs of ErbBs (Kokai *et al.*, 1989; Alimandi *et al.*, 1995; Wallasch *et al.*, 1995).

In addition to the receptor level, combinatorial signaling by the ErbB network is further diversified at two additional levels. First, multiple EGF-like ligands exist and they differentially induce certain receptor combinations (Pinkas-Kramarski *et al.*, 1996b), probably because each ligand carries not only a high affinity site, but also a 'low affinity/broad specificity' site that recruits the dimer's partner (Tzahar *et al.*, 1997). Interestingly, some ligands induce more potent signals than others although they bind to the same receptor. For example, on certain cellular systems, such as keratinocytes (Barrandon and Green, 1987) and endothelial cells (Schreiber *et al.*, 1986), TGFα is more potent than EGF, although both ligands bind to ErbB-1 with comparable affinity (Kramer *et al.*, 1994). Another level of signal diversification is comprised of the multiple substrates of RTKs; members of this large group of SH-2 domain-containing proteins are differentially recruited to certain ErbBs. Examples include the phosphatidylinositol 3′-kinase and c-Cbl that preferentially engage with ErbB-3 (Soltoff *et al.*, 1994) and with ErbB-1 (Levkowitz *et al.*, 1996), respectively. Despite differences in second messenger activation, signaling by all ErbBs feeds into the MAPK pathway, raising the question of how signal specificity is maintained intracellularly. One potential answer is provided by results obtained with other growth factors in pheochromocytoma cells, indicating that the kinetics of MAPK activation, and especially its inactivation, may critically determine signal identity (reviewed in Marshall, 1995). Unlike the activation process which has been extensively studied, the inactivation phase of RTK signaling is poorly understood. One obvious candidate is the process that leads to endocytosis, down-regulation and degradation of ligand-activated receptors. Indeed, individual ErbB proteins differ remarkably in their rate of endocytosis and down-regulation (Baulida *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996a).

Our present study addressed the hypothesis that the multiple ligands of ErbBs differ in their potencies because they differentially recruit certain heterodimeric receptor combinations (Beerli and Hynes, 1996; Pinkas-Kramarski *et al.*, 1996b; Gulliford *et al.*, 1997). To this end we compared signaling by EGF and TGFα, a pair of ligands that display respectively weak and strong signaling in most tissues, in a well-defined cellular system expressing combinations of ErbB-1 with either ErbB-2 or ErbB-3. In contrast to our working hypothesis, differences in potency were observed even in the absence of either co-receptor, namely ErbB-2 or ErbB-3. However, to our surprise, the co-receptors potentiated the effect of EGF without significantly affecting TGFα signaling. In subsequent experiments we investigated the mechanism of potentiation and found that the co-receptors, by forming heterodimers with ErbB-1, redirected this receptor to an endocytic route that allows receptor recycling and, therefore, enhanced signaling. These results imply that EGF-like ligands whose ErbB specificity is shared are functionally distinct, and suggest that alternative endocytic routing may be critical for controlled inactivation and fine-tuning of signal transduction.

Results

ErbB-2 and ErbB-3 potentiate EGF mitogenicity but not TGFα signaling

To examine possible functional relationships between the multiplicity of EGF-like ligands and the extensive inter-receptor interactions within the ErbB family of receptors we used the two best characterized ligands of the family, namely EGF and TGFα, in combination with a series of cell lines co-expressing ErbB-1 with either ErbB-2 (D12 cells), or with ErbB-3 (D13 cells) (Pinkas-Kramarski *et al.*, 1996a). A third cell line that singly expresses ErbB-1 (D1 cells) was used for comparison of ErbB-1 homodimers with heterodimers of this receptor. In addition, a chimeric EGF/TGFα molecule, designated E4T, comprised of the A and B loops of EGF, and the C loop of TGFα, was used because of its superior mitogenic activity to that of other chimeric molecules and the parental ligands (Lenferink *et al.*, 1997). Due to their dependence on interleukin-3 (IL-3), the cell lines we employed are extremely sensitive to EGF-like ligands when tested in the absence of IL-3. Thus, TGFα exerted mitogenic stimuli that were at least 10-fold more active than EGF-induced signals when tested on D1 cells (Figure 1A). However, E4T was even more potent in inducing cell proliferation. This pattern of relative potency was also reflected in long-term survival experiments in which IL-3 was replaced by the corresponding ErbB-1 ligand and cell survival monitored daily (Figure 1B). Introduction of ErbB-2 into D1 cells elevated the basal proliferation rate of the resulting cell line, D12, in agreement with previous reports (Kokai *et al.*, 1989; Cohen *et al.*, 1996; Tzahar *et al.*, 1996; Zhang *et al.*, 1996). Thus, whereas maximal stimulation of D1 cells by IL-3 was 5.5-fold, only a 2-fold activation was displayed by D12 cells. Interestingly, however, co-expression of ErbB-2 together with ErbB-1 (D12 cells) resulted in remarkable potentiation of the mitogenic action of EGF; whereas half maximal mitogenic effect was induced by 10 ng/ml of this ligand on D1 cells, only 0.7 ng/ml was necessary to stimulate the D12 cells (Figure 1A, compare D1 with D12 panels). In contrast, ErbB-2 co-expression only slightly improved the mitogenic action of TGFα and E4T. In fact, in the presence of ErbB-2, EGF almost approached the high mitogenic activity of TGFα, a phenomenon that was reflected, in part, also in a long-term survival assay (Figure 1B, D12 panel). Interestingly, ErbB-3 only partially potentiated EGF activity in D13 cells (compare the EC_{50} of EGF on D13 cells, which is 2 ng/ml, with that on D1 cells, which is 10 ng/ml). Once again, co-expression exerted no significant effect on the potency of either TGFα, or E4T (D13 panels in Figure 1). In conclusion, ErbB-2, and to some extent also ErbB-3, specifically enhance the EGF-induced mitogenic action of ErbB-1, probably by forming heterodimeric complexes with this receptor.

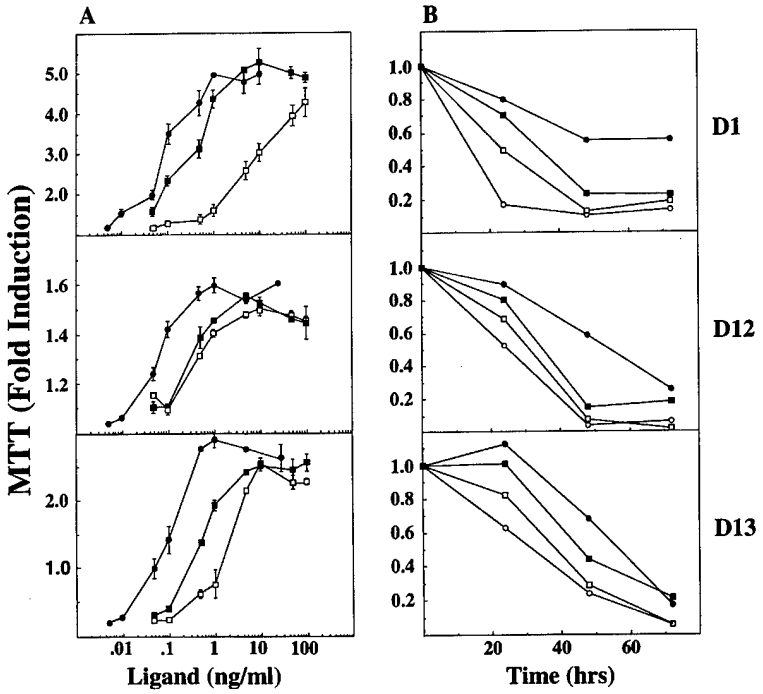

Fig. 1. Ligand-induced proliferation and survival of ErbB-expressing 32D-cells. (**A**) The following derivatives of 32D cells were examined for cell proliferation by using the MTT assay: D1 cells that singly express ErbB-1, D12 cells expressing a combination of ErbB-1 with ErbB-2, and D13 cells expressing a combination of ErbB-1 with ErbB-3. Cells were washed free of serum factors and IL-3, and seeded at a density of 5×10^5 cells/ml in RPMI-1640 medium containing serial dilutions of EGF (□), TGFα (■), or E4T (●). Following 24 h of incubation, the MTT assay was performed as described in Materials and methods. (**B**) The indicated sublines of 32D-cells were plated as described above in the presence of 100 ng/ml EGF, TGFα or E4T [symbols are as in (A)]. Cell proliferation was measured daily using the MTT assay. As a negative control cells were plated in serum- and IL-3-free medium (○). The data from both experiments are given as the means of three determinations. Bars in (A) represent standard deviations. The experiments were repeated three times. The responses to IL-3 (fold induction) of D1, D12 and D13 were 5.54±0.63, 1.96±0.67 and 3.03±0.81, respectively.

Binding parameters may explain superiority of E4T, but not the difference between EGF and TGFα

Perhaps the simplest explanation for the observed differences in mitogenic potencies of EGF, TGFα and E4T might be parallel differences in receptor binding affinities. To examine this possibility we labeled the three ligands with ^{125}I and determined their apparent binding affinities to D1, D12 and D13 cells using ligand displacement analysis. The results of this experiment are shown in Figure 2A. Evidently, the apparent affinities of EGF, TGFα and E4T were not remarkably different when tested on D1 cells, in agreement with a similar analysis that was performed with fibroblasts (Lenferink *et al.*, 1997). Co-expression of ErbB-2 (or ErbB-3) only slightly improved the affinity of D12 cells (or D13 cells) to EGF or TGFα (Figure 2A, D12 and D13 panels). Notably, ligand binding assays performed with derivatives of 32D cells usually yield affinities that are consistently lower than those measured with adherent cell types such as fibroblasts or epithelial cells. For example, the K_d values of EGF and TGFα binding to adherent cells are in the range of 0.1–5 nM (Tzahar *et al.*, 1994; Lenferink *et al.*, 1997), whereas D1 cells bind these ligands with apparent K_d values of 30–50 nM. This may be due to the relatively prolonged washing procedure required in the case of the 32D myeloid cells, which results in an overall reduction in assay sensitivity. We used a ligand dissociation assay as an alternative to partly overcome this limitation. Cells were loaded with the various radiolabeled ligands under saturating conditions, then the unbound ligand was removed and the rates of release of radioactivity were monitored. Clearly, the rates of release of E4T from the surfaces of all three cell lines examined were higher than the dissociation rates of EGF and TGFα (Figure 2B). In addition, the co-expressed co-receptors, namely ErbB-2 and ErbB-3, comparably decelerated the rate of dissociation of EGF and TGFα from ErbB-1, in agreement with previous reports (Kokai *et al.*, 1989; Karunagaran *et al.*, 1996; Tzahar *et al.*, 1996). Taken together, rapid dissociation from the cell surface may be involved in the mitogenic superiority of E4T over EGF and TGFα. However, neither the enhancement of EGF signaling by the co-receptor, nor the superiority of TGFα over EGF may be attributed to binding parameters.

Co-receptors decelerate ligand depletion and internalization, but clearance of the E4T superagonist is defective

Because E4T is released from the cell surface at a much faster rate then EGF or TGFα, we expected that these latter ligands would be depleted from the medium at a much faster rate than E4T. This possibility was tested by incubating D1, D12 and D13 cells with serial dilutions of the ligands for 24 h, thereby allowing their depletion from the medium. Then we determined the relative concentration of each ligand in the conditioned medium by employing a bioassay that uses serum-starved HER-14 fibroblasts overexpressing ErbB-1. As predicted, the rate of ligand depletion inversely correlated with mitogenic potency; the weakest and the strongest mitogens of D1 cells, namely

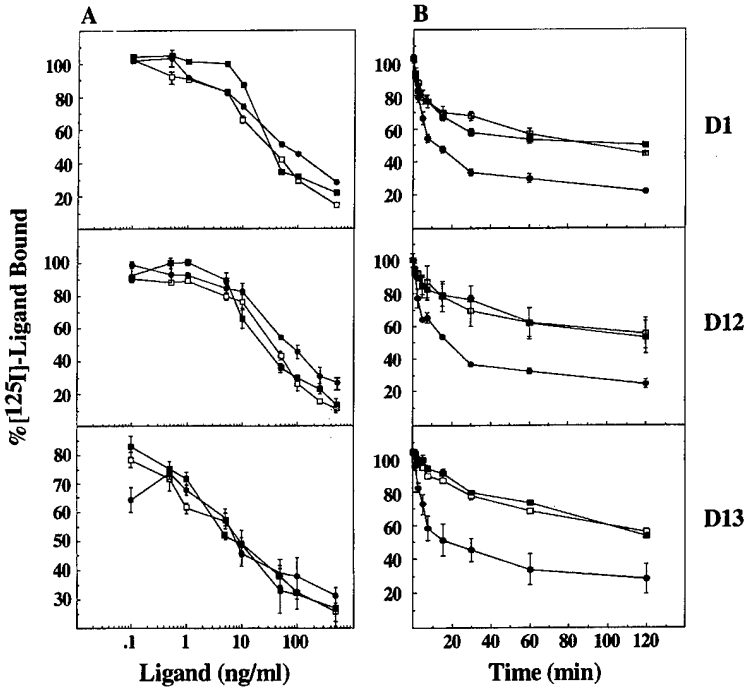

Fig. 2. Ligand displacement and dissociation analyses. (**A**) Displacement analysis was performed with 1.0×10^6 cells of the indicated subclones of the 32D cell line. Cells were washed free of IL-3 and serum factors using binding buffer, and subsequently incubated for 2 h at 4°C with [^{125}I]EGF (1 ng/ml) in the presence of serial dilutions of unlabeled EGF (□), TGFα (■) or E4T (●). Unbound ligand was removed by sedimenting the cells through a cushion of calf serum. The results are presented as the mean ±SD of two determinations. Experiments were repeated three times with similar results. (**B**) The indicated cell lines were incubated for 2 h at 4°C with [^{125}I]EGF (□), [^{125}I]TGFα (■), or [^{125}I]E4T (●), each at 60 ng/ml. Then, the unbound ligand was replaced by an excess of the unlabeled growth factor (3 μg/ml), and cell-bound radioactivity was monitored at the indicated time intervals. Results are expressed as the fractional ligand binding (mean ±SD) relative to the amount of ligand that bound at $t=0$. The experiment was performed in duplicate and repeated twice with similar results.

EGF and E4T, respectively displayed rapid and slow depletion from the medium (Figure 3A). For example, when D1, D12 and D13 cells were incubated for 24 h with a low concentration of EGF (1 ng/ml) and the resulting conditioned media compared with medium similarly incubated in the absence of cells, we observed a 63, 28 and 47% reduction, respectively, in mitogenic activity. The corresponding numbers for TGFα were 28, 36 and 43%, and for E4T, 14, 16 and 24%. Thus, the presence of ErbB-2 significantly decelerated the rate of EGF depletion, but it less efficiently affected removal of E4T or TGFα from the medium. The relative rates of cell-mediated removal of the three ligands correlated with their mitogenic potency, implying that an endocytic mechanism is responsible for the observed differences in signaling potency. Consistent with this model, co-expression of the less potent co-receptor, ErbB-3, together with ErbB-1 only partly extended the half life of EGF (D13 panel in Figure 3A).

To test directly a model involving endocytosis, we comparatively analyzed the internalization rates of the various ligands of ErbB-1, and also determined their dependence on the presence of a co-receptor, either ErbB-2 or ErbB-3. It is notable that our previous experiments, which used a standard ligand internalization assay, detected only minor differences between the rates of ligand internalization through homo- and hetero-dimeric receptors (Pinkas-Kramarski *et al.*, 1996a). Therefore, we tested several ligand internalization protocols for their ability to discriminate between the rates of endocytosis of homo- and hetero-dimeric receptors and selected the following assay. Cells were first incubated in the cold with a moderately low concentration of the respective radiolabeled ligand, then the unbound ligand was removed, cells chased at 37°C with a saturating ligand concentration and the ligand distribution between the cell surface and the cytoplasm was determined using an acid wash. This protocol differs from that previously employed (Pinkas-Kramarski *et al.*, 1996a) in two aspects. First, a 10-fold lower ligand concentration was used in order to avoid saturation of the coated pit-mediated internalization pathway (reviewed in Sorkin and Waters, 1993). Secondly, other protocols do not include a step that removes unbound ligand prior to initiation of endocytosis. Therefore, continuous uptake of the radiolabeled ligand may mask differences in endocytosis rates. The results of this experiment presented in Figure 3B confirmed that internalization of E4T is significantly slower than that of EGF or TGFα. More importantly, the rate of EGF uptake was remarkably decelerated by a co-expressed ErbB-2, but less so in the presence of ErbB-3 (EGF panel in Figure 3B). The rate of TGFα internalization was similarly affected by the presence of ErbB-2 or ErbB-3 (hTGFα panel in Figure 3B), implying that receptor heterodimers endocytose more slowly than homodimers, irrespective of ligand identity. Because both homodimers and heterodimers of ErbB-1 apparently exist in D12 and in D13 cells, the net kinetics of heterodimer internalization is expected to be even slower than the rates reflected in Figure 3B. Taken together, the data presented in Figure 3 suggest that signaling superiority of E4T is due to the slow rates of internalization and clearance of this ligand from the medium. Possibly,

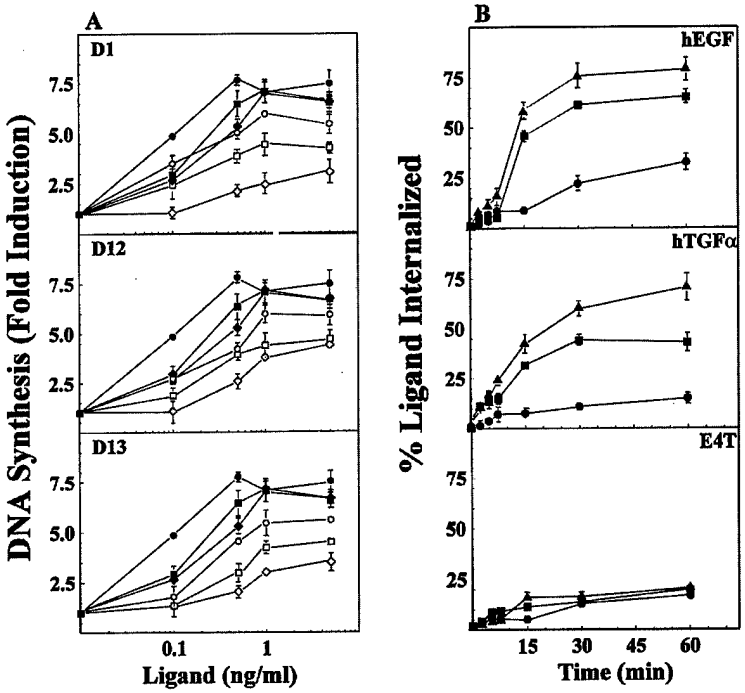

Fig. 3. Receptor-mediated depletion and uptake of ligands. (**A**) Increasing concentrations of the following ligands were incubated for 24 h at 37°C with the indicated derivatives of 32D cells (open symbols): EGF (diamonds), TGFα (squares) or E4T (circles). For control, ligands were similarly incubated in the absence of cells (closed symbols). The capacity of the resulting conditioned media to stimulate DNA synthesis in HER-14 fibroblasts was then determined as described in Materials and methods. Results are given as the mean ± SD of three individual experiments carried out in duplicate. (**B**) For determination of ligand internalization rates, radiolabeled forms of the indicated ligands (each at 1 ng/ml) were incubated for 2 h at 4°C with the following derivatives of 32D cells: D1 (▲), D12 (●) or D13 cells (■). Following incubation on ice, cells were washed free of unbound ligand and incubated at 37°C for various time intervals with excess of the corresponding unlabeled ligand (at 3 μg/ml). Cellular uptake of radioactivity was monitored by removing surface-bound ligand with an acidic ligand-strip buffer. Data are presented as the mean ± SD of duplicate determinations. Each experiment was repeated at least twice.

rapid dissociation of E4T from ErbB-1 (Figure 2) prevents efficient internalization. On the other hand, the relatively weak signaling capacity of EGF through the singly expressed ErbB-1 is attributed by our results to the efficient rate of cellular uptake of this ligand. Moreover, the potentiating effect of ErbB-2 is probably due to its ability to decelerate both the rate of internalization (Figure 3B) and the rate of clearance of EGF from the medium (Figure 3A), in line with the relatively slow down-regulation and endocytosis of ErbB-2 (Sorkin *et al.*, 1993; Baulida *et al.*, 1996). Despite these consistencies, our results cannot provide a satisfactory explanation for the relatively high potency of TGFα; although this ligand is more potent than EGF on D1 cells, and it is almost equipotent to EGF on D12 cells (Figure 1), its rates of internalization (Figure 3B), depletion from the medium (Figure 3A) and dissociation from the cell surface (Figure 2B), are only slightly different than those of EGF, and they apparently cannot account for the EGF-specific 10–15-fold mitogenic enhancement effect of ErbB-2 (Figure 1A).

EGF and TGFα are comparably degraded, but E4T degradation is limited

According to one possibility, EGF and TGFα are similarly endocytosed, but whereas the former is efficiently degraded in lysosomes, the other escapes intracellular degradation. To test this model we treated cells with each of the radiolabeled ligands under conditions that prevent receptor recycling and retard targeting to the degradative pathway. Upon transfer of chilled cells to 37°C ligand degradation was allowed and monitored using acid precipitation. The results presented in Figure 4 indicate that E4T is degraded at a slower rate than EGF and TGFα, as expected on the basis of its slower rate of uptake (Figure 3B), but intracellular degradation of EGF and TGFα were comparable in kinetics and extent. Remarkably, expression of a co-receptor together with ErbB-1 only slightly affected the rates of ligand degradation. In experiments not shown we confirmed a previous report (Hamel *et al.*, 1997) that degradation of both ligands was significantly inhibited by chloroquine, a drug known to inhibit degradation in both endosomal (prelysosomal) and lysosomal compartments, but leupeptin, a tripeptide whose inhibitory action is specific to lysosomes (Cardelli *et al.*, 1989), did not affect TGFα degradation. Conceivably, EGF is destined for lysosomal degradation after endocytosis (Renfrew and Hubbard, 1991), whereas TGFα is degraded in a non-lysosomal compartment whose identity is only partly characterized (Hamel *et al.*, 1997). Independent of its exact intracellular location, endocytic degradation of EGF and TGFα cannot provide an explanation for the superiority of TGFα and the potentiating effect of ErbB-2.

The presence of a co-receptor specifically increases acid sensitivity of EGF binding

It is well established that binding of EGF and TGFα (Ebner and Derynck, 1991), as well as binding of various chimeras of these two ligands (Lenferink *et al.*, 1997), display differential sensitivity to acidic pH. This, in turn, is thought to allow recycling of TGFα-bound receptors to

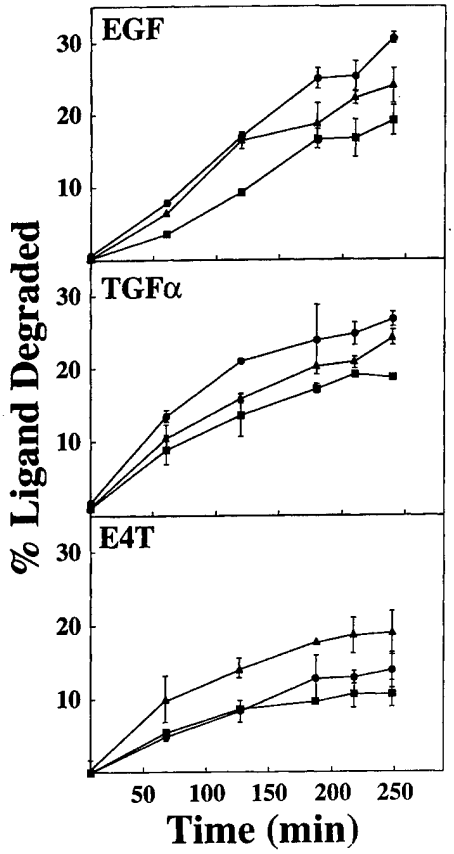

Fig. 4. Kinetics of ErbB-mediated ligand degradation. The indicated radiolabeled ligands (each at 1 nM) were incubated for 1 h at 20°C with the following derivatives of 32D cells: D1 (▲), D12 (●) or D13 cells (■). Thereafter, the cells were spun through a cushion of serum to remove unbound ligand, and then incubated at 37°C for various time intervals. Media were then collected and cells solubilized. The fraction of acid-soluble (degraded) ligand in the medium was determined by counting the acid-soluble radioactivity in the medium and the total cell-associated radioactivity. The results are expressed as the average percentage of acid-soluble radioactivity, relative to the sum of cell-associated and medium-released radioactive counts. Bars represent standard deviations. The experiment was performed in duplicate and repeated twice.

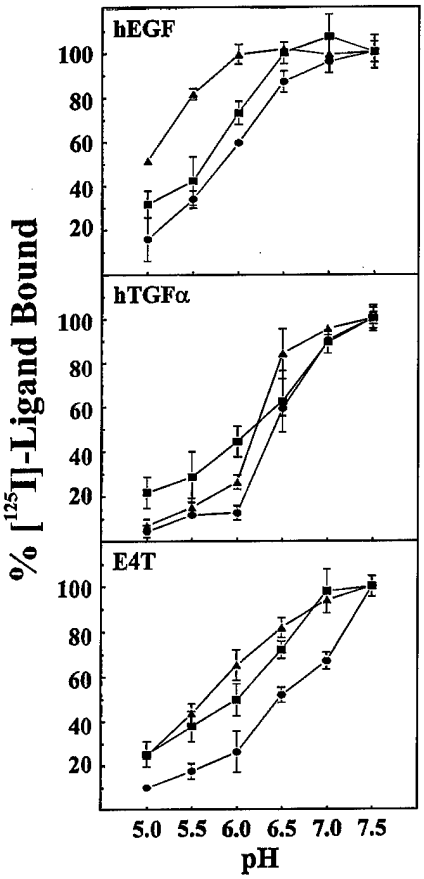

Fig. 5. pH sensitivity of ligand binding to specific combinations of ErbBs. D1 (▲), D12 (●) or D13 cells (■) were incubated for 2 h at 4°C with radiolabeled forms of the indicated ligands (each at 60 ng/ml). The pH of the binding buffer was adjusted to the indicated values. Unbound radioactivity was removed by sedimenting the cells through a cushion of calf serum, prior to γ-counting. Results are shown as the mean ± SD of a triplicate experiment which was repeated twice.

the cell surface, thereby augmenting TGFα biological action (Ebner and Derynck, 1991). On the other hand, because EGF resists the moderately acidic pH of early endosomes, this ligand does not permit receptor recycling, and the ligand–receptor complex is destined for degradation in lysosomes. To examine the possibility that the presence of a co-receptor alters pH sensitivity of ligand binding, we analyzed the interaction between EGF, TGFα and E4T with D1, D12 and D13 cells under various pH conditions. In line with previous observations, EGF binding to ErbB-1 displayed remarkable stability when compared with TGFα and E4T (Figure 5). However, the presence of a co-receptor, either ErbB-2 or ErbB-3, significantly destabilized these interactions. By contrast, the co-receptors only slightly affected the relatively sensitive binding of TGFα (hTGFα panel in Figure 5). In addition, a moderate effect of the co-receptors was observed in the case of E4T (Figure 5). On the basis of these observations we predict that the lysosome-destined EGF-driven ErbB-1 is re-routed to recycling back to the cell surface once a co-receptor is present. On the other hand, co-expression of ErbB-2 or ErbB-3 may not alter routing of a TGFα-driven ErbB-1, because this ligand rapidly dissociates in early endosomes regardless of the dimerization state of its receptor.

EGF-driven homodimers of ErbB-1 are degraded, but heterodimers are recycled to the cell surface

To monitor the fate of ErbB-1 after ligand-induced endocytosis, we induced down-regulation of this receptor using an unlabeled ligand and then determined the status of the remaining surface-associated binding sites by performing a radio-receptor assay. The results of this experiment revealed that ErbB-1 was destined for different fates depending on the activating ligand; upon EGF binding ErbB-1 rapidly disappeared from the surface of D1 cells, but both TGFα and E4T caused re-appearance of binding sites following an initial phase of receptor down-regulation (Figure 6). That re-appearance was due to recycling of endocytosed receptors was indicated by its complete inhibition by monensin (Figure 6, right column), a drug known to inhibit recycling of transmembrane receptors (Basu *et al.*, 1981), including the EGF-receptor (Gladhaug

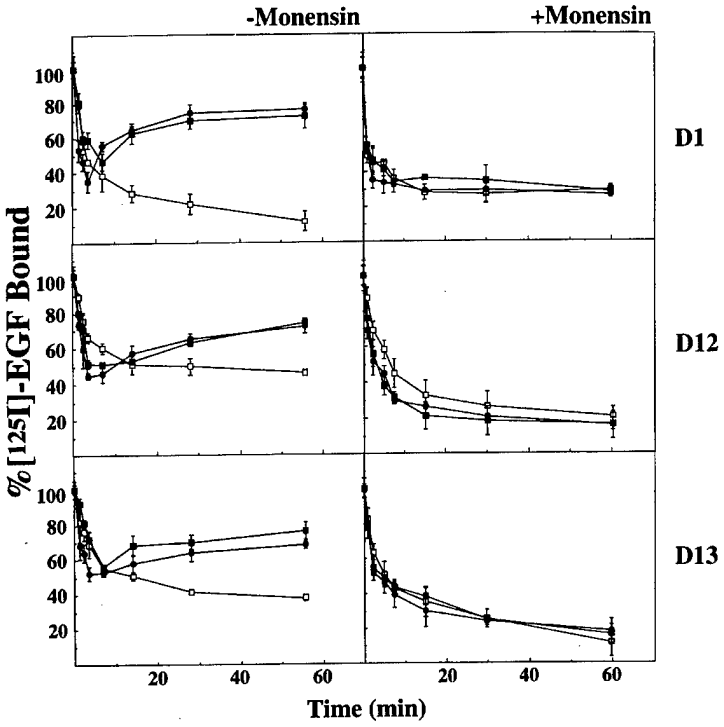

Fig. 6. Dependence of down-regulation and recycling of ErbB-1 on ligand identity and receptor interactions. The indicated derivatives of 32D cells (1.0×10^6 cells per each data point) were incubated for 2 h at 4°C with the following ligands (each at 60 ng/ml): EGF (□), TGFα (■) or E4T (●), in the absence (left panels) or presence (right panels) of monensin (0.3 mM). The cells were then transferred to 37°C and incubated for the indicated time intervals. The residual level of surface receptor that did not undergo down-regulation was determined by performing a direct binding assay with radiolabeled EGF. The results are calculated as the fraction of the initial binding of [^{125}I]EGF at $t=0$, and are presented as the mean ± SD. The experiment was performed in duplicate and repeated twice.

and Christofferson, 1988). It is worthwhile noting, however, that monensin may affect other intracellular processes. For example, it has been reported that treatment with monensin can inhibit the addition of N-linked oligosaccharide chains to ErbB-1 (Mayes and Waterfield, 1984). The patterns of receptor down-regulation exhibited by EGF-treated D12 and D13 cells were different; whereas the behavior of TGFα- or E4T-driven receptors was not significantly altered by either co-receptor, in the presence of either ErbB-2 or ErbB-3 the EGF-induced down-regulation was decelerated and eventually reached a relatively high steady state (D12 and D13 panels in Figure 6). This effect was more pronounced in the case of D12 cells, in correlation with the observation that ErbB-2 potentiates EGF signaling better than does ErbB-3 (Figure 1). The relatively high steady-state of ErbB-1, that was induced by the presence of ErbB-2 or ErbB-3, was completely abolished by monensin (Figure 6). The absence of net re-appearance of binding sites, following an initial drop, in the case of EGF-treated D12 and D13 cells is attributed to the combined contribution of homodimers (that are destined for degradation) and heterodimers (that are destined for recycling). Thereby, heterodimer formation can alter the endocytic fate of an EGF-driven ErbB-1 from degradation to recycling. This scenario is consistent with the observation that the two co-receptors destabilized EGF binding at moderately acidic conditions (Figure 5), and they also attenuated both the rate of EGF uptake (Figure 3B) and the rate of ligand disappearance from the growth medium (Figure 3A).

EGF and TGFα similarly recruit ErbB-2, but engagement of ErbB-3 by heterodimerization is limited

The specificity of the potentiating effect of ErbB-2 to EGF action, but not to the biological effect of TGFα, may be explained by an alternative model which argues that TGFα less efficiently recruits ErbB-2 into heterodimers with ErbB-1 (Gulliford *et al.*, 1997), and therefore its action is unaffected by the presence of the co-receptor. Two experimental strategies were employed in order to test the validity of this model. First, the ability of TGFα to induce heterodimers was compared with that of EGF by covalent labeling of ErbB-1 with either ligand and determination of the extent of co-precipitation of the co-receptor (either ErbB-2 or ErbB-3) with ErbB-1. The results of this experiment indicated that EGF- and TGFα-labeled monomers (M) and dimers (D) of ErbB-1 underwent comparable co-immunoprecipitation by antibodies directed to ErbB-2 (Figure 7A), in agreement with recent reports (Beerli and Hynes, 1996; Riese *et al.*, 1996). The interaction between ErbB-3 and ErbB-1 was hardly detectable by this assay (D13 lanes in Figure 6B), confirming weak stability of the ErbB-1/ErbB-3 complex (Tzahar *et al.*, 1996). Thus, recruitment of a co-receptor cannot explain the differences between EGF and TGFα, because these ligands similarly engage ErbB-2 heterodimerization. This conclusion was independently supported by a second approach using monoclonal antibodies (mAbs) to ErbB-2, denoted L26 and L140, that respectively inhibit or only slightly affect heterodimer formation

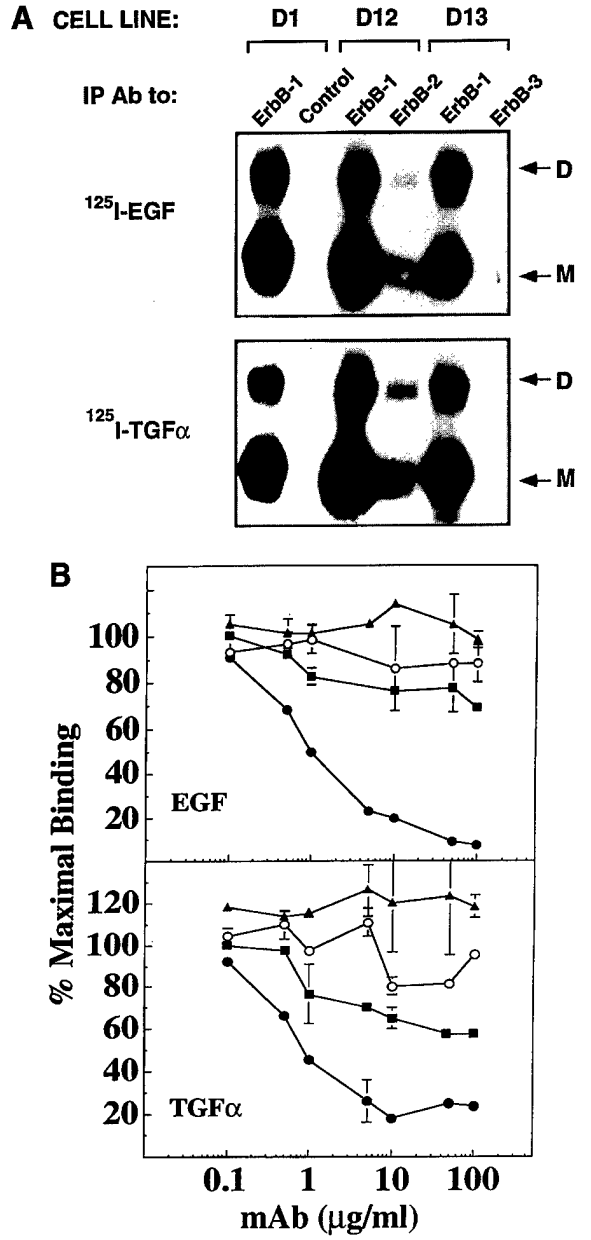

Fig. 7. Ligand-induced formation of ErbB-1-containing heterodimers. (**A**) D1, D12 and D13 cells were incubated with radiolabeled EGF or TGFα (each at 20 ng/ml) for 90 min at 4°C. Covalent crosslinking was performed by further incubation for 1 h with the bivalent crosslinking reagent BS^3. Cell lysis and immunoprecipitation (IP) of the indicated ErbB proteins were then performed and followed by gel electrophoresis. The resulting autoradiograms are shown, along with the locations of monomeric (M_r ~180 kDa) and dimeric (D) ligand–receptor complexes. (**B**) D12 cells were incubated for 2 h at 4°C with either [^{125}I]EGF or [^{125}I]TGFα (each at 10 ng/ml), along with the indicated concentrations of the following anti-ErbB-2 mAbs: L26 (■) and L140 (○). For positive control we used a neutralizing antibody to ErbB-1, mAb 528 (●). As a negative control we used a mAb to a hepatitis B antigen (▲). Binding of the radiolabeled ligands was determined as described under Materials and methods and presented as the mean ±SD of three determinations. The experiment was repeated three times with similar results.

(Klapper *et al.*, 1997). Since by breaking ErbB-2-containing heterodimers these mAbs partly reduce the binding of ligands to their direct receptors (Klapper *et al.*, 1997), ligand binding may be used as a readout of ErbB-2 recruitment into heterodimers. When tested on D12 cells, mAb L26 and to some extent also mAb L140 reduced binding of EGF and TGFα (Figure 7B), implying that both ligands can induce formation of the ErbB-1/ErbB-2 heterodimeric complex. Of note, in these cells TGFα was inhibited more efficiently than EGF. For control, a ligand-competitive mAb to ErbB-1 was used and it reached an almost complete inhibition of both ligands, but an irrelevant mAb was inactive (Figure 7B). Taken together, the results presented in Figure 7 exclude the possibility that differences in heterodimer recruitment account for the EGF-specific potentiating action of a co-receptor, thus strengthening an endocytosis-based mechanism of signal potentiation.

Discussion

Previous analyses concentrating on the relative mitogenic and transforming abilities of ErbB proteins and their ligands established the notion that cells co-expressing ErbB-1 together with ErbB-2 are more effectively transformed than either cells expressing ErbB-1 alone (Kokai *et al.*, 1989), or ErbB-1 in combination with ErbB-3 (Cohen *et al.*, 1996). Likewise, TGFα was shown to be more mitogenic and transforming than EGF in an autocrine or paracrine context (reviewed in Salomon *et al.*, 1995). Our present study links the superiority of receptor heterodimers with ligand specificity and provides a mechanistic basis for this functional linkage. After dealing with the proposed mechanism of signal potentiation, we discuss below the implications of our findings to current open questions, such as the extent of physiological redundancy of the multiple EGF-like ligands and the role of ErbB-2 in cancer.

The observation that ErbB-2 can *trans*-potentiate the proliferative effect of EGF more efficiently than ErbB-3 is best interpreted in terms of heterodimer formation: ErbB-1/ErbB-2 interactions are more prevalent than ErbB-1/ErbB-3 associations (Figure 7A) (Tzahar *et al.*, 1996). Nevertheless, EGF is known to activate ErbB-3 in cells overexpressing ErbB-1 (Kim *et al.*, 1994; Soltoff *et al.*, 1994), and phosphorylation of ErbB-3 apparently takes place within an EGF-driven ErbB-1/ErbB-3 heterodimer (Riese *et al.*, 1995; Pinkas-Kramarski *et al.*, 1996a; Zhang *et al.*, 1996). Thus, the relatively weak interactions between ErbB-1 and ErbB-3 may explain why the potentiating effect of ErbB-3 is weaker than that of ErbB-2 (Figure 1A). Assuming a heterodimerization model, we propose that the three ligands we tested utilize distinct mechanisms for signal potentiation. These mechanisms are described below.

EGF

According to our results, EGF can signal through two alternative pathways that are schematically presented in Figure 8. In the absence of a co-receptor, EGF is rapidly endocytosed, and due to the relatively stable binding to ErbB-1 it resists the low pH of early endosomes (Figure 5). This targets homodimeric complexes of ErbB-1, along with EGF, to degradation in lysosomes (Figure 4), and results in an almost complete disappearance of surface ErbB-1 (Figure 6). On the contrary, in the presence of a co-receptor the ternary complex (EGF, ErbB-1 and the co-receptor), whose internalization rate is relatively slow

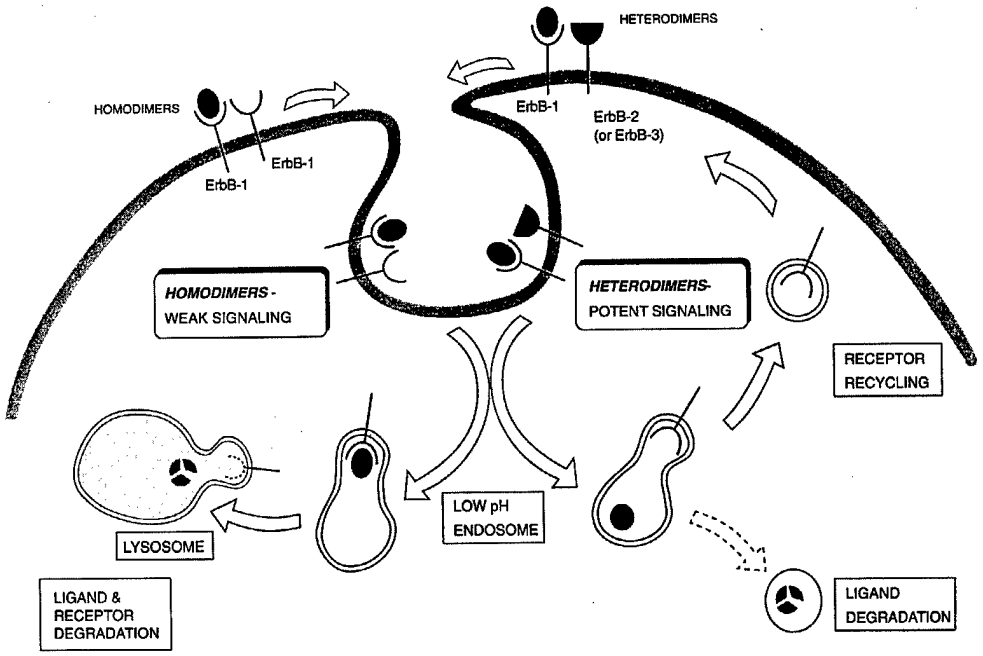

Fig. 8. Proposed endocytic model of heterodimerization-mediated tuning of mitogenic signals. EGF-occupied homodimers of ErbB-1 are destined for rapid endocytosis and lysosomal degradation that efficiently terminate signaling. In the presence of ErbB-2 (or ErbB-3), EGF signals are enhanced because ErbB-1/ErbB-2 heterodimers release EGF when the pH of early endosomes decreases. This allows recycling of the receptor back to the cell surface, thereby augmenting EGF signaling. Not presented are the pathways undertaken by TGFα and E4T. Whereas the former directs ErbB-1 to recycling regardless of the presence of a co-receptor, E4T signaling is further enhanced by its rapid on/off rates of interaction with ErbB-1. Both routes of EGF/ErbB-1 endocytosis result in intracellular degradation of the ligand, either because co-existence of homo- and hetero-dimers allows inter-pathway leakage of ligand molecules, or because the recycling route is coupled to non-lysosomal proteolytic degradation.

(Figure 3B), dissociates under the moderately acidic conditions of early endosomes (Figure 5), and consequently ErbB-1 recycles back to the cell surface (Figure 6). The exact fate of the two other molecular components of the ternary complex is unclear; whereas the co-receptor either escorts ErbB-1 to the plasma membrane, or undergoes enhanced degradation (Worthylake and Wiley, 1997), degradation of EGF takes place in an unknown compartment, probably the same non-lysosomal vesicular compartment that processes TGFα (Hamel *et al.*, 1997). Nevertheless, some recycling of undegraded EGF molecules seems to occur, as the rate of depletion of this ligand from the medium is decelerated in the presence of a co-receptor (Figure 3A). Regardless of the exact fate of their molecular components, the altered endocytic routing of ErbB-1-containing complexes may be responsible for signal potentiation, because this pathway constantly delivers unoccupied ErbB-1 molecules to the plasma membrane. By contrast, in the case of a homodimeric ErbB-1, efficient down-regulation of the receptor takes place and, therefore, signaling is short lived. It is relevant that a linkage between defective internalization of ErbB-1 and strong proliferative signals has been previously established by using an endocytosis-impaired mutant of this receptor (Wells *et al.*, 1990).

TGFα

Because binding of this ligand to both homo- and heterodimeric complexes of ErbB-1 is pH-sensitive (Figure 5), TGFα directs receptor recycling regardless of the presence of a co-receptor (Figure 6). Consequently, receptor down-regulation (Figure 6) and ligand depletion (Figure 3A) are slower in the case of TGFα than they are with EGF, which may explain the stronger mitogenic signal of TGFα, as compared with EGF (Figure 1). In a parallel set of experiments that examined neuregulin signaling through the extremely potent ErbB-2/ErbB-3 complex we found that the cellular routing of neuregulin-driven ErbB-3 is similar to that of TGFα-driven ErbB-1 complexes (Waterman *et al.*, 1998), implying that recycling of ErbBs is a common mechanism of signal potentiation. Interestingly, however, the cellular context may affect intracellular routing of TGFα as human endometrial and other cells display more rapid processing of this ligand relative to EGF, and this correlates with biological potency (Korc and Finman, 1989; Reddy *et al.*, 1996b).

E4T

Unlike EGF and TGFα which differ only slightly in binding parameters (Figure 2), examination of the rate of dissociation of the chimeric superagonist E4T revealed a relatively high rate of release from both homo- and hetero-dimeric receptor complexes (Figure 2B). This was confirmed using plasmon resonance to measure in real time the association and dissociation rates of the three ligands from a soluble form of ErbB-1; E4T was found to behave differently to EGF and TGFα, in having both a relatively high association and dissociation rate constant (A.E.G.Lenferink and M.D.O'Connor-McCourt, manuscript in preparation). This kinetic combination may explain why the apparent affinity of E4T is similar to that of EGF or TGFα (Figure 2A). In addition, E4T displayed several significant landmarks, such as relatively slow rates of endocytosis (Figure 3B) and intracellular degradation (Figure 4), combined with pH-sensitive receptor binding (Figure 5), and an ability to induce receptor recycling

(Figure 6). It is relevant that a mutant form of EGF, denoted EGF-Val-47, shares with E4T resistance to intracellular degradation and high biological potency (Walker *et al.*, 1990). Collectively, the biochemical features of E4T appear to contribute to high signaling potency in the following way: due to its rapid on/off kinetics, E4T only transiently stimulates its receptor and therefore this ligand causes inefficient endocytosis. Moreover, due to their pH sensitivity, those E4T-bound ErbB-1 molecules that eventually undergo endocytosis rapidly recycle back to the cell surface, probably along with the chimeric ligand. Thus, the relatively strong mitogenic signal of E4T may be entirely due to inefficient signal inactivation processes. An alternative interpretation emerged from a study performed with a chimeric ligand similar to E4T (Puddicombe *et al.*, 1996). Like E4T, the other chimera displayed superagonist activity and its rate of depletion from the growth medium was relatively low. However, it has been noted that activation of receptor autophosphorylation by this ligand was more sustained than by EGF, and its mitogenic superiority displayed cell type specificity, suggesting a contextual requirement.

A central issue of the above described models of signal potentiation is the assumption that heterodimer formation by ErbB-1 can affect intracellular routing of this receptor. Most likely heterodimers do not dissociate upon endocytosis, thereby allowing an '*in trans*' effect of the co-receptor on the rate and destination of receptor endocytosis. It has been shown previously that the rates of ligand internalization and receptor down-regulation are high in the case of ErbB-1 and relatively low in the case of ErbB-2, ErbB-3 and ErbB-4 (Baulida *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996a). Because ErbB-3 is practically devoid of tyrosine kinase activity (Guy *et al.*, 1994), and a kinase-defective mutant of ErbB-1 displays altered routing (Glenney *et al.*, 1988; Felder *et al.*, 1990), it is understandable why ErbB-3-containing heterodimers are less efficiently endocytosed. In fact, our recent results indicate that ErbB-3 undergoes slow endocytosis, which is followed by rapid recycling to the cell surface (Waterman *et al.*, 1998), a route that is apparently shared with a kinase-defective mutant of ErbB-1. On the other hand, the slow endocytic rates of ErbB-2 and ErbB-4 are more difficult to reason. One potential explanation may involve their inability to recruit components of the coated pit, such as the adapter protein 2 (Baulida *et al.*, 1996), which are necessary for rapid internalization. Alternatively, signals inhibitory for rapid internalization may reside in the structurally distinct cytoplasmic portions of the co-receptors (Sorkin *et al.*, 1993).

What is the physiological role of *trans*-potentiation through heterodimer formation? An evolutionary perspective may provide a hint to the answer; while only one EGF-like ligand and one ErbB-like receptor exist in worms (Kornfeld, 1997), several dozen ligands and four receptors are known in mammals. This evolutionary expansion of the number of distinct components was probably aimed at increasing physiological versatility. One such mechanism emerges from the present study: controlled expression of a co-receptor may confer superior signaling properties to others. By inference, the multiple ligands of ErbB-1 may not have redundant functions; within the appropriate context of a receptor and a co-receptor some ligands may be superior to others. An example from mammals may demonstrate the issue: whereas normal hepatocytes respond to TGFα better than to EGF (Guren *et al.*, 1996), their embryonic counterparts respond equally well to the two ligands (Lipeski *et al.*, 1996), in accordance with the presence of ErbB-2 in fetal cells (W.E.Russell, personal communication) but not in adult hepatocytes (Carver *et al.*, 1996).

The biochemical mechanism underlying the prognostic value of ErbB-2 in human cancer is currently unclear (Hynes and Stern, 1994; Stancovski *et al.*, 1994). According to an autonomous type of mechanism, ErbB-2 contributes to high proliferation and tissue invasion perhaps because its direct ligand, whose identity is unknown, activates homodimeric ErbB-2 complexes in a manner similar to an oncogenic rat mutation (Weiner *et al.*, 1989). Alternatively, an overexpressed ErbB-2 is oncogenic perhaps because the basal tyrosine kinase activity of this receptor is relatively high (Lonardo *et al.*, 1990). The non-autonomous type of mechanism (Tzahar and Yarden, 1998) implies that ErbB-2 functions solely as a molecular amplifier of signaling initiated by all stromal EGF-like ligands (Karunagaran *et al.*, 1996), because this receptor is the preferred heterodimeric partner of all ErbB proteins (Tzahar *et al.*, 1996; Graus-Porta *et al.*, 1997), and its coupling to the MAPK pathway is extremely efficient (Ben-Levy *et al.*, 1994). The realization that ErbB-2 is a slowly internalizing receptor that can *trans*-potentiate EGF signaling by decelerating the relatively fast rate of ErbB-1 endocytosis (Figure 8) suggests that ErbB-2 supports oncogenesis not only by decelerating the rate of growth factor dissociation from heterodimeric receptor complexes (Karunagaran *et al.*, 1996), but also by delaying their inactivation process. One immediate implication is that ErbB-2 overexpression in carcinomas may be related to the type of stromal ligands expressed in the vicinity of each particular tumor. Likewise, this mechanism may be critical in metastasis; successful seeding of ErbB-2-overexpressing tumor cells at selected sites may be determined by the presence of ligands whose action is potentiated by the co-receptor. Establishment of this and other predictions made on the basis of the *trans*-potentiation effect of ErbB-2 will require additional studies.

Materials and methods

Materials, buffers and antibodies

Human recombinant EGF and TGFα were obtained from Boehringer Mannheim. Binding buffer contained RPMI-1640 medium supplemented with 0.5% bovine serum albumin (BSA). mAbs L26 and L140 raised against the extracellular part of the human ErbB-2 receptor were as described (Klapper *et al.*, 1997). mAb 528 directed against the extracellular domain of ErbB-1 was a kind gift of John Mendelsohn (MD Anderson Cancer Center, TX). The acidic ligand-strip buffer (pH 2.5) contained 5 mM acetic acid, 2.5 mM KCl, and 135 mM NaCl. Solubilization buffer contained 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM Na_3VO_4, 5 μg/ml pepstatin A, 5 μg/ml leupeptin and 5 μg/ml aprotinin. HNTG buffer contained 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol.

Mutant growth factor production

The chimeric growth factor E4T, consisting of EGF sequences N-terminal to the fourth cysteine of the EGF-like motif and TGFα sequences C-terminal to this cysteine, was constructed as described (Kramer *et al.*,

1994), cloned into the pEZZ18 expression vector (Pharmacia, Uppsala, Sweden) (van de Poll *et al.*, 1995) and harvested as a secreted protein A-containing product from the periplasmic space of *Escherichia coli* KS474, a protease-deficient mutant (Strauch *et al.*, 1989). Bacteria were grown overnight in 2YTE medium under continuous agitation (200 r.p.m.). The fusion protein was isolated as described (Nilson and Abrahmsen, 1990) and purified using IgG–Sepharose (Pharmacia). Protein yield was determined by using a binding competition assay with biotin-labeled protein A (van Zoelen *et al.*, 1993). E4T was enzymatically cleaved from protein A by factor X digestion and separated by an additional run over an IgG column. Final purification of the sample was done by reverse-phase chromatography as described previously (van de Poll *et al.*, 1995). Fractions of 1 ml were collected and tested for binding to HER-14 cells (Lenferink *et al.*, 1997). The quantity of E4T was calculated using the peak area representing the binding activity at 229 nm in the chromatography profile. Murine EGF from a natural source was used under the same experimental conditions as a standard (van de Poll *et al.*, 1995).

Cell culture

32D murine myeloid cells (Greenberger *et al.*, 1983), transfected with the various combinations of erbB-encoding plasmid or viral vectors (Pinkas-Kramarski *et al.*, 1996a) were grown in RPMI-1640 medium supplemented with antibiotics, 10% heat-inactivated fetal bovine serum and 0.1% medium conditioned by IL-3-producing X63/0 cells (Karasuyama and Melchers, 1988). Cells were kept under continuous selection using 0.4 mg/ml hygromycin B (Boehringer Mannheim) for D1 cells and additionally 0.6 mg/ml G418 (Boehringer Mannheim) for D12 and D13 cells. NIH 3T3 cells transfected with the wild-type human EGF receptor (HER-14 cells) and expressing 4.0×10^5 ErbB-1 molecules/cell (Honegger *et al.*, 1988), were cultured in gelatinized flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum.

Ligand displacement assays

The rationale of this assay was adopted and modified from a previous protocol (Reddy *et al.*, 1996a). Essentially, ligand concentration profiles were determined in media conditioned by preincubation with ErbB-expressing cells. Because a radioimmunoassay and a radio-receptor assay were less satisfactory, we used a bioassay with HER-14 murine fibroblasts overexpressing ErbB-1. Recombinant human EGF, TGFα and the chimera E4T were radiolabeled using the indirect Iodogen method (Pierce, Roxford, IL), as described previously (Peles *et al.*, 1993). For ligand displacement analysis, 1.0×10^6 cells were washed once with binding buffer, incubated with a radiolabeled ligand (at 1 ng/ml) for 2 h at 4°C in 0.2 ml of the same buffer, containing serial dilutions of the unlabeled ligand. To terminate ligand binding, cells were sedimented (9000 *g*, 2 min), washed once with 0.5 ml binding buffer and loaded on top of a 0.7 ml cushion of BSA. Tubes were spun again to remove the unbound ligand and radioactivity in the cell pellets was counted directly.

Cellular proliferation assays

To analyze ligand-induced proliferative responses of D1, D12 and D13 cells, 5.0×10^4 cells were washed free of IL-3, resuspended in RPMI-1640 and seeded in 96-wells plates. For dose–response experiments, serial dilutions of a ligand were added in RPMI-1640 medium and cells were incubated for 24 h at 37°C. IL-3 (1:1000 of medium conditioned by a producer cell line) was used as a positive control. Proliferation was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which determines mitochondrial activity in living cells (Mosman, 1983). During an incubation for 2 h at 37°C with MTT, living cells transform the tetrazolium ring into dark blue formazan crystals which can be quantified by reading the optical density at 540–630 nm after lysis of the cells with acidic 2-propanol. For cell survival experiments, cells were seeded at the same density in 96-well plates and incubated at 37°C with a fixed ligand concentration (100 ng/ml). Cell survival was determined 24, 48 and 72 h after ligand addition using the MTT method.

Ligand depletion assay

The rationale of this assay was adopted and modified from a previous protocol (Reddy *et al.*, 1996a). Essentially, ligand concentration profiles were determined in media conditioned by preincubation with ErbB-expressing 32D cells. Because radioimmunoassay and radio-receptor assay was less satisfactory we used a bioassay with HER-14 fibroblasts overexpressing ErbB-1. HER-14 cells were seeded in gelatinized 24-well dishes (1.8 cm^2) at a density of 6.0×10^4 cells/well in 1 ml DMEM/10% serum. After 24 h of incubation the medium was replaced by 0.9 ml of DMEM/Ham's F12 medium (1:1) supplemented with 30 nM Na_2SeO_3, 10 μg/ml human transferrin and 0.5% BSA. After an additional incubation for 48 h, 0.1 ml medium that was conditioned for 24 h by D1, D12 or D13 cells was added. Eight hours later 0.5 μCi [^{3}H]thymidine (TdR) was added in 0.1 ml Ham's F12 medium. Incorporation of the tracer into cellular DNA was determined 24 h after growth factor addition as described previously (van Zoelen *et al.*, 1986).

Receptor recycling assays

To quantify receptor recycling, 1.0×10^6 cells were incubated for 2 h at 4°C with various ligands (at 60 ng/ml) in the absence or presence of 0.3 mM monensin (as indicated), and then transferred to 37°C for various time periods. Subsequently, cells were sedimented (9000 *g*, 2 min), resuspended, and incubated in ice-cold ligand-strip buffer for 2 min on ice. Cells were sedimented again, neutralized in binding buffer and incubated in the same buffer for an additional 1 h at 37°C to allow intact internalized receptors to recycle to the cell surface. To quantify the number of ErbB-1 molecules on the cell surface, cells were incubated for 2 h at 4°C with [^{125}I]EGF, sedimented as above, rinsed once in binding buffer and spun through a serum cushion to remove the unbound ligand, prior to γ-counting.

Ligand internalization assays

The fate of various ligands was determined by incubating 32D cells (1.0×10^6 cells) with 1 ng/ml radiolabeled EGF, TGFα or E4T. Following 2 h at 4°C cells were washed in binding buffer, resuspended in the same buffer that contained unlabeled ligand (3 μg/ml) and transferred to 37°C for the indicated time periods. Then, cells were immediately cooled on ice, incubated for 5 min in the acidic ligand-strip buffer (pH 2.5), and sedimented through a serum cushion. The released ligand was considered as cell surface-associated ligand. Cells were lysed in 1% Triton X-100 for 1 h at room temperature prior to γ-counting.

Ligand dissociation assays

Dissociation of radiolabeled human EGF, TGFα and E4T was investigated using 1.0×10^6 D1, D12 or D13 cells. Cells were rinsed once in binding buffer and subsequently incubated (2 h, 4°C) with excess (60 ng/ml) radiolabeled ligand in binding buffer. Then, the tubes were spun and the cell pellet was resuspended and incubated at 4°C in binding buffer supplemented with 3 μg/ml unlabeled ligand for the indicated time spans. Finally, cells were pelleted and lysed in 100 mM NaOH containing 0.1% sodium dodecylsulfate prior to γ-counting.

Ligand degradation assays

Derivatives of 32D cells (1.0×10^6 cells) were washed free of IL-3 and subsequently incubated at 20°C for 60 min with radiolabeled ligand (at 1 nM) in binding buffer. Then, cells were spun through a serum cushion to remove the unbound ligand and incubated, without ligand, for up to 240 min at 37°C. At various time points, trichloroacetic acid-precipitable counts in the medium (degraded ligand) were determined.

Ligand crosslinking analyses

For chemical crosslinking experiments with 32D cells, 5.0×10^6 cells were incubated for 2 h on ice with 20 ng/ml radiolabeled EGF or TGFα. The chemical crosslinker bis(sulfonylsuccinimidyl)-suberate (BS^3, Pierce, Roxford, IL) was added to a final concentration of 1 mM. Cells were then incubated for 45 min at 4°C and subsequently washed with phosphate buffered saline, pelleted by centrifugation, and lysed in solubilization buffer. Lysates were cleared by centrifugation, and immunoprecipitated with antibodies against specific ErbB proteins. Rabbit antibodies were directly coupled to protein A–Sepharose beads while shaking (1 h, 4°C); mouse antibodies were coupled indirectly using rabbit-anti-mouse IgG under the same conditions. ErbB proteins present in the cell lysate were immunoprecipitated with the protein A–Sepharose-antibody complex for 2 h at 4°C. Precipitates were washed three times in HNTG buffer prior to heating for 5 min at 95°C in gel sample buffer under reducing conditions. Samples were analyzed using gel electrophoresis (7.5% acrylamide).

Acknowledgements

We thank Sara Lavi for technical assistance and John Mendelsohn for the 528 mAb to ErbB-1. A.E.G.L. is a recipient of an EMBO Fellowship (ASTF8588). This work was carried out with financial support from the Department of the Army (grant DAMD17-97-1-7290), The National

Cancer Institute (grant CA72981) and The Israel Science Foundation administered by the Israel Academy of Sciences and Humanities.

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Received December 29, 1997; revised March 26, 1998; accepted April 20, 1998